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Original Article

Characterization and SNP genotyping in Selenoprotein genes (Sep15 and DIO1) of *Bubalus bubalis*

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ABSTRACT

Selenium (Se) is an essential trace element for animals and humans. It is found in soil and water and consequently enters the food chain through the root ways of plants and aquatic organisms. Selenium exerts its biological function largely through selenoproteins which contain selenium in the form of selenocysteine. About 25 different selenocysteine-containing selenoproteins have so far been observed in human which can be grouped into five distinct families. In the present study, we focused on Sep15 and DIO1 gene in *Bubalus bubalis*. Sep15 codes for antioxidant enzyme or protein that may control the quality of cellular proteins and is expressed mostly in secretory tissues like liver, kidney, prostrate and thyroid. DIO1 is expressed mainly in liver, thyroid gland and adipose tissue and its main function is to convert tetraiodothyronine (T4) to its active form thyroxine (T3) in the presence of deiodinases enzyme. The main aim of the study was to characterize these two genes and to find out the buffalo specific SNPs. Sep15 gene sequenced for 2532 bases with 5 exons and DIO1 gene sequenced (3773 bases) consisted of 4 exons. In Sep15 and DIO1, 8 and 10 SNPs, respectively were identified and 3 SNPs in each gene were non synonymous. These SNPs are novel and reported for the first time in Indian buffalo and has a potential for their use in diversity analysis and association with various selenium related traits.

Keywords : Selenoprotein genes, SNPs, DIO1, Sep15, *Bubalus bubalis.*

INTRODUCTION

The water buffalo, *Bubalus bubalis* holds tremendous potential in livestock sector in many Asian countries, particularly India. The Buffalo is backbone of the farmer's economy benefiting nearly half of humanity in over 40 countries. The domestic water buffalo is a major source of milk, meat, draught, hide and employment to the marginal farmers and landless labourers in many Asian countries. Buffaloes are the mainstay of Indian dairy industry playing a major role, by contributing approximately >50% of total milk production. Among the 13 well defined river buffalo breeds of India (www.nbagr.res.in) buffaloes especially from Northern and Western parts of the country have been traditionally evolved for high milk production. India has approximately 110 million buffalo, primarily of the river type, representing fifty-six percent of the total world buffalo population.

DNA markers are playing an increasingly important role in animal breeding. It has been estimated that there is on an average 0.5 to 1.0 heterozygous SNPs per 1000 base pairs in the human genome (Mark, 2001). In livestock, one expects a slightly lower number of SNPs because of intensive selection in their breeding programmes but the number of SNPs still exceeds other types of genetic markers (Ramesha *et al*., 2002). The abundance of SNPs in the genome makes them a powerful tool for genetic studies.

Selenium is metalloid element that is essential to human and animal health in trace amount but is harmful in excess. The selenium status of plants and animals varies markedly around the world as a result of different geological conditions. High selenium concentrations are associated with some phosphatic rocks, organic rich black shales, coals and sulphide mineralization whereas most other rock types contain very low concentrations. Selenium deficiencies occurs in farm animals over much larger areas of the world including much of United States than does selenium toxicity (Muth *et al*., 1967 National research council 1971)

Globally selenium deficient soils are more widespread than selenifereous soils. Animal health is affected by selenium deficiency and excess in diet, the intake of selenium being dependent on the amount of selenium taken up by plants as bioavailable selenium. Because of its vital role, deficiency of selenium in animals may result in wide variety of clinical signs. Selenium in the form of selenoproteins is critical in the formation of hormones and other endocrine systems and their roles in control of intracellular redox environment, cellular growth and defense against oxidative stress. A major sign of pronounced selenium deficiency is "White muscle disease" also known as "Nutritional muscular dystrophy" which usually occurs in young calves (Ammerman and Miller, 1975). Selenium responsive unthriftness varying from sub clinical growth depression to progressive loss of condition usually

associated with diarrhoea can occur in cattle of all ages. Likewise fertility in females may be adversely affected. In cows, the inclusion of supplemental selenium in cow-feeds, has substantially decreased incidence of retained placenta (Julien *et al.,* 1976).

Among the selenoproteins, identified in mammals, are four glutathione peroxidases, three thioredoxin reductases, three thyroid hormone deiodinases, selenophosphate synthetase, selenoprotein P, selenoprotein W, selenoprotein T, selenoprotein R (also called selenoprotein X), selenoprotein N and a 15-kDa selenoprotein (Gladyshev *et al*., 1999, Burk *et al*., 1999, Kryukov *et al*., 1999, Lescure *et al*., 1999).

Sep15 codes for antioxidant enzyme or protein that control the quality of cellular proteins. Sep15 is expressed mostly in secretory tissues like liver, kidney, prostrate and thyroid. The gene coding bovine Sep15 is mapped on BTA3 corresponding to BBU6 (Amaral *et al.,* 2008). The position of selenocysteine is conserved in the Sepl5 vertebrate sequences, whereas in insects and nematodes, Sec is replaced with Cys. Homology analyses also indicate that vertebrate Sep15 sequences are highly conserved (Vadim Gladyshev *et al*., 2001). For example, mature human and mouse Sepl5 differ by only three amino acid residues but in bovines it differs by 7 amino acid residues with respect to humans.

Thyroid hormones play a critical role in mammalian development and metabolism. Their activity is regulated in a complex, tissue-specific manner by three isoforms of deiodinases. It was demonstrated that thyroid hormone administration can increase milk production in dairy cows. DIO1 is expressed predominantly in thyroid, liver and kidney. The iodothyronine deiodinases catalyze the removal of an iodine residue from the pro-hormone thyroxine (T4) molecule, thus producing either the active form triiodothyronine (T3 activation) or inactive metabolites (reverse T3; inactivation). DIO1 is also located on BBU6 corresponding to BTA3 in cattle. **Present study was undertaken to characterize and identify polymorphism in Sep15 and DIO1 genes in buffalo and to develop protocol for genotyping SNPs in these two genes.**

MATERIALS AND METHODS

The genomic DNA samples from 24 animals drawn from 5 diverse Indian water buffalo (Murrah, Toda, Pandharpuri, Banni, Chilika) and swamp buffalo were isolated using freshly collected blood samples using protocols given by Sambrook *et al*., 2001. Murrah are North Indian buffaloes while Toda belongs to Nilgiri hills of Tamil Nadu. Pandharpuri belongs to Western regions, Banni is extensively

found in Gujarat while Chilika belongs to eastern regions of India. Swamp buffalo samples from Manipur were also collected for the present study.

The exonic regions were amplified by designing overlapping primers in exon-intron boundaries for both Sep15 and DIO1 genes. These primers were designed from sequence available in Bovine Ensembl Transcript for Sep15 (ENSBTA00000023372) and DIO1 (ENSBTAT00000061123). All PCR primers were designed using web interface of Primer3. PCR primers for amplification of both the selenoprotein genes are given in Table 1. 25 µl reaction containing 50ng of sample DNA, 5pmol of each primer, 200mM of each dNTP, 1U Taq DNA polymerase (Sigma Aldrich) with 1X PCR buffer with 1.5 mM $MgCl_2$ was utilized. Amplification reactions were carried out in Eppendorf (Pro–S) with program as 5 min denaturation at 94°C followed by 35 cycles of 94°C for 45 s, annealing (58° - 62°C) for 45 s and extension at 72°C for 45s, with a final extension of 10 min at 72°C. The PCR products were visualized on 2% agarose gel with 100bp ladder. Prior to sequencing the PCR products were purified with Exo SAP (New England Biolabs, Beverely, MA, USA) enzymatic digestion. Sequencing was carried out with a final volume of 10 µl using ABI Big Dye chemistry with manufacturers' protocol on 3130XL genetic analyzer (Invitrogen). Each product was sequenced using reverse and forward primer. The sequences were aligned using BIOEDIT and Clustal W alignment software. The sequences were translated to get respective amino acid sequences for the genes.

Table 1 The primer pairs used for amplification of genomic DNA

SNo.	Gene	Forward primer	Reverse primer	Size
1.	Sep15_1	CCGTAGTTAATGCCCGTCTC	CAGGTCAGAAGAAGGAACTGC	428 bp
2.	Sep15_2	AATTTAAAAATTGTCTCCAAATGAGG	GTCTATAACCATAATTTTCCCCTTCA	500 bp
3.	Sep15_3	AGGCCTTCATTTTCTCTTCTGA	CCATTACTGTTTGCTGTCAACC	418 bp
4.	Sep15_4	GTCTTTCCAATATGGGATATCTTTC	GGACGATGGAGATGGGAGTA	380 bp
5.	Sep15_5	TTAAGCTAGATTGAATTGGTTTGG	AAAAAGGGTTTTGTTAACTTCTTACC	400 bp
6	DIO1_1	CTGGCACCTTCCTTCTGTCT	CAGAAAAGGAGCAGCTGGAG	569 bp
7	DIO1_2	GCCTCTGCTAAATCGTGTGTT	GAAGCACTCCAGGTTTCTGC	507 bp
8	DIO1_3	AGCCCCCACAACAAGAATTA	ATTTCATCCCCTGTGAGGTG	502 bp
9	DIO1_4	TCTGCTTTTCCTCCTGCTTC	CCCCATGCTTAGTTCTAAAAGC	686 bp

The gene sequences were aligned taking *Bos taurus* as reference using BIOEDIT sequence alignment tool. PCR-RFLP protocols were developed for SNPs detected for each gene. Enzyme restriction sites for the recognition of the SNPs were identified and primer pairs were designed from buffalo gene sequence to amplify the flanking regions of SNPs. PCR products were subjected to digestion by restriction enzyme in a total volume of 10 µl (8 µl of PCR product, 1X of enzyme buffer, 1 unit of enzyme) and placed in ABI thermocycler at their respective temperatures for overnight incubation. After digestion, the digested products were visualized by 2.5% agarose (Promega, USA) gel electrophoresis.

RESULTS AND DISCUSSION

The 5 primers designed for Sep15 and 4 primers for DIO1 gene (Table 1) from the Ensembl database yielded PCR products of expected sizes. This shows that there is not much difference between cattle and buffalo in these 2 genes and there was complete transferability of primer sequences. The sequences of both the genes were submitted to Genbank with accession number ((JQ041636) for Sep15 gene and accession number (JQ791197) for DIO1 gene respectively.

The Sep15 consisted of 5 exons (Fig 1) and our sequence consisted of 2532 bases. The sequence consisted of 5'UTR region (142 bases) 3'UTR (182 bases) alongwith partial introns and gaps. The Sep15 gene codes for a protein of 162 amino acids. The protein Id for the gene is AFA26564.2. A total of 8 SNPs were detected in Sep15 gene with 24 diverse buffalo samples. 3 SNPs in exonic region at position G→A (R16Q), T→G (L18R) and C→G (T25R) were non-synonymous. The nucleotide position with respect to Accession number JQ041636 and the change in amino acid are given in Table 2.

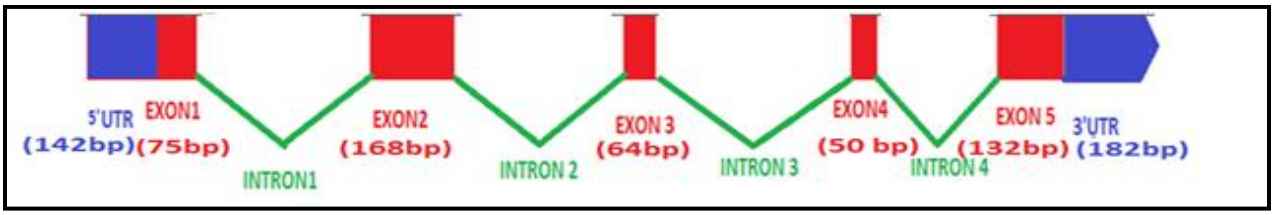

Fig 1 Structure of Sep15 gene showing exons introns and UTR regions

Table 2 Buffalo specific SNPs in Sep15 gene

S.No.	Position (in bp)	SNP	Amino acid change
1.	189bp	A/G*	R16Q
2.	195bp	T/G*	L 18R
3.	216bp	C/G*	T25R
4.	257bp	G/T	
5.	259bp	C/G	
6.	643bp	C/T	
7.	867bp	A/G	
8.	2388bp	A/C	

* non synonymous

The DIO1 sequenced (3773 bases) for the present study consisted of 4 exons, partial introns and gaps. 5'UTR of 123 bases and 3'UTR of 60 bases was also sequenced (Fig 2). The gene codes for a total of 249 amino acids. The protein Id for the gene is AFE86483. Out of a total of 10 SNPs, 3 SNPs in the exonic regions were non-synonymous (A→G (H22R), C→G (T31R) and G→A (V146I). The nucleotide position with respect to Accession no. JQ791197 and the change in amino acid are given in Table 3.

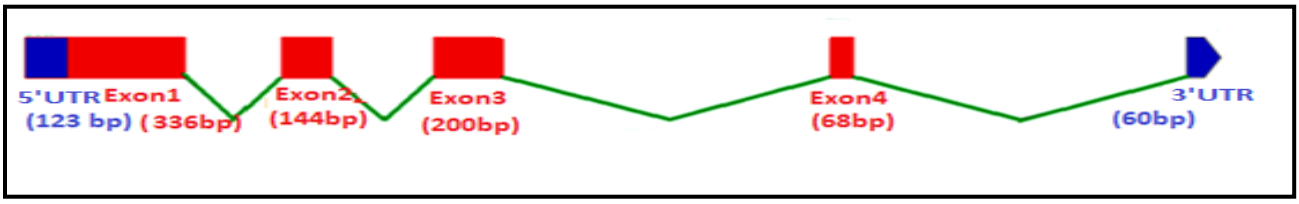

Fig 2 Structure of DIO1 gene showing exons introns and UTR region

Table 3 Buffalo specific SNPs in DIO1 gene

S.NO	Position (in bp)	SNP	Amino acid change
1.	112bp	G/T	
2.	188bp	A/G*	H22R
3.	215bp	C/G*	T31R
4.	941bp	G/A*	V146I
5.	1110bp	A/T	

6.	1527bp	A/G	
7.	2164bp	A/G	
8.	2210bp	A/G	
9.	2240bp	A/G	
10.	2305bp	C/G	

*non synonymous

Sep15 and DIO1 genes were genotyped using PCR-RFLP in 40 samples of five recognized breeds of Indian water buffalo and swamp buffalo. For Sep15, enzyme (HpyCh4V) was selected which recognized the SNP (189A/G).

With the help of restriction digestion of the PCR product of exon1 with HpyCH4V, GG genotype resulted in three bands at 113, 127, 186bp and AA resulted in four bands at 113, 186, 53 and 74bp. The heterozygous AG samples produced five bands of 113, 127, 186, 53 and 74 bp (Fig.3A). The genotype frequency was calculated for different breeds as given in Table 4.

Similarly, for DIO1 gene, two enzymes Nsp1 and HgaI were selected which resulted in only one cutting site in the PCR product of exon1 for 188 A/G and 215 C/G respectively

The AA genotype was uncut while genotype GG resulted in two bands 186 and 382bp when digested with Nsp1 enzyme for 188 A/G SNP. The heterozygous AG samples produced three bands of 568, 382 and 186bp (Fig. 3B). The genotype frequency was calculated for different breeds as given in Table 5.

215 C/G SNP digested with Hga1 enzyme GG genotype was uncut and CC genotype resulted in two bands 215 and 353 bp. Heterozygous CG samples produced three bands of 568, 215 and 353bp (Fig. 3C). The genotype frequency was calculated for different breeds as given in Table 6.

Table 4 Genotype frequency of Sep15 gene with Hpych4V Locus

Breed	GG	AG	AA
Murrah	0.777	0.055	0.111
Pandharpuri	0.891	0.026	0.105
Toda	0.911	0.058	0.028
Banni	0.869	0.043	0.086
Chilika	0.629	0.000	0.370
Manipuri	0.400	0.000	0.600

Table 5 Genotype frequency of DIO1 gene with Nsp1 locus

Breed	AA	AG	GG
Murrah	0.562	0.437	0.000
Pandharpuri	0.628	0.342	0.028
Toda	0.000	0.875	0.125
Banni	0.131	0.763	0.105
Chilika	0.000	0.250	0.750
Manipuri	0.000	0.500	0.500

Table 6 Genotype frequency of DIO1 gene with Hga1 locus

Breed	CC	CG	GG
Murrah	0.051	0.923	0.02
Pandharpuri	0.258	0.129	0.612
Toda	0.114	0.371	0.514
Banni	0.166	0.433	0.400
Chilika	0.250	0.250	0.500
Manipuri	0.266	0.200	0.530

CONCLUSION

Sepl5 was discovered by virtue of the presence of selenium in the protein and the relative abundance of Sepl5 compared to other selenoproteins. Dietary selenium is perhaps receiving more importance as the selenium content affects the animal health both in deficiency as well as excess. *Ratala et al*., 1990 highlighted the selenium toxicity to be one of the major causes of Khari disease in buffalo population of Baitadi district. The majority of livestock producing areas of the world are subject to the multifaceted effects of selenium on livestock health and disease. The genomic sequences for Sep15 and DIO1 gene are reported for the first time in *Bubalus bubalis.* SNPs in exonic region were novel and these SNPs can further be used to associate the selenium deficiency and toxicity in buffaloes.

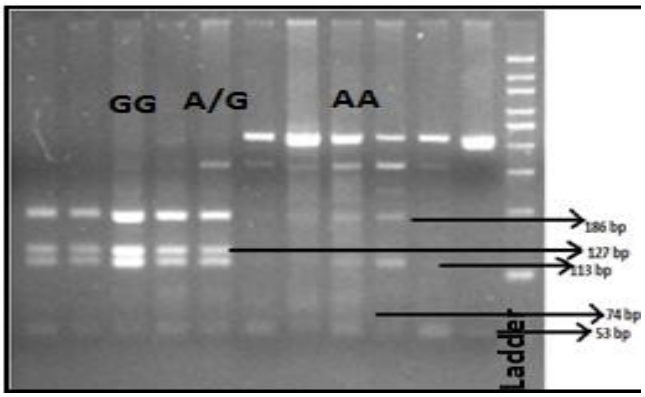

Fig. 3A PCR-RFLP of Sep15 gene with HpyCH4V enzyme

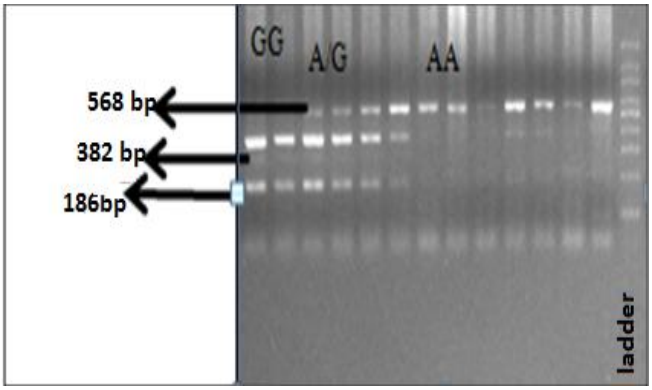

Fig. 3B PCR-RFLP of DIO1 gene with Nsp1 enzyme

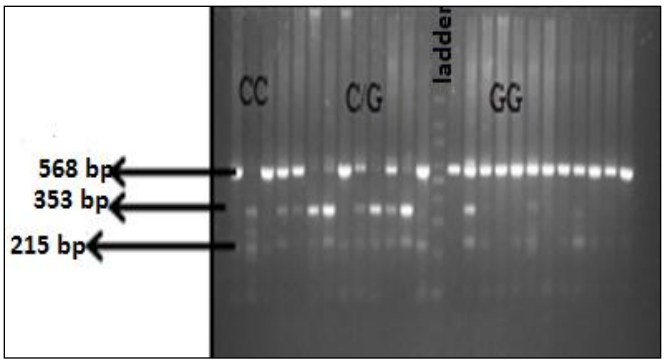

Fig. 3C PCR-RFLP of DIO1 gene with Hga1 enzyme

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