GENETIC POLYMORPHISM OF AQUAPORIN 7 GENE AND ITS ASSOCIATION WITH SEMEN QUALITY IN SURTI BULLS

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ABSTRACT

The present study was carried out with the aim to characterise Aquaporin 7 (AQP7) gene and to investigate the association of genetic variants with semen quality in Surti bulls. A total of 21 Surti bulls maintained at Centralized Semen Collection Centre, Dharwad, Karnataka were included in the study. Single Nucleotide Polymorphisms (SNPs) in the coding region of AQP7 gene were identified through polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing. A total of 14 different SSCP variants were found in exonic regions along with the exon-intron boundaries (amplicons) of AQP7 gene. Statistical analysis was done by SAS 9.3. Analysis of exons (amplicons) 1, 4, 5, 7 and 8 revealed two, three, four, three and two unique SSCP band patterns, respectively. SSCP variants of exon 1 were found to have significant ($P \leq 0.05$) effect on Hypo-osmotic swelling reactivity in frozen semen. SSCP variants of exons 5 and 8 were found to have significant ($P \leq 0.01$) effect on sperm viability in fresh semen and Post thaw motility, respectively. The study indicated high degree of genetic variability in AQP7 gene in Surti bulls. The observed SSCP variants having association with semen parameter shows the possibility of using AQP7 gene as a candidate gene for identification of markers for semen quality traits in buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, AQP7 gene, PCR-SSCP, polymorphism, semen quality, surti bulls

INTRODUCTION

Bull fertility and semen quality are very important for the bovine industry. Quality of semen can be used as a physiological indicator of fertility in bulls (Miglior 1999). Artificial insemination (AI) is one of the most important methods for the genetic improvement of semen quality traits in dairy herds (Parmentier *et al*., 1999, Qing *et al*., 2013). The key purpose of frozen semen preparation is harvesting the maximum amount of high quality sperm from genetically superior bulls for its use in AI. But fertility traits are less heritable and expressed later in life which hinder the improvement of bull fertility using traditional selection methods. Molecular DNA markers can

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be used for genetic improvement, through marker assisted selection. Single stranded conformation polymorphism (SSCP) technique followed by sequencing of the amplicon can unveil single nucleotide polymorphism (SNP) which may explain the variability at the phenotypic level (Fujita and Silver, 1994).

Aquaporin 7 (AQP7) gene, a member of aqua-glyceroporins functions in the rapid transport of glycerol and water (Borgnia *et al*., 1999; Maeda *et al*., 2008; Sohara *et al*., 2009). It is located on chromosome 8 of bovine (BTA 8), has 7 coding exons and 1 non-coding exon. It is of 16.25 kilobase (kb) pairs. The AQP7 gene is expressed at the tail of spermatids, spermatozoa and anterior tail portion of ejaculated sperm (Suzuki-Toyota *et al*., 1999). Suzuki-Toyota *et al.* (1999) reported its role in spermatogenesis. It maintains sperm quality through sperm maturation by transporting glycerol, urea and other small non-electrolytes from epididymal fluid and also acts as effective cryoprotectant (Sales *et al*., 2013). Candidate genes having major effect on semen quality traits would be beneficial as a marker towards selection of semen quality traits. Surti buffalo are used for upgrading local buffaloes in Gujarat, Rajasthan and Karnataka. They are medium-sized rusty brown in colour and their home tract is in Anand, Kheda and Vadodara districts of Gujarat. Now a day, demand of semen doses for AI is increasing. In India, buffaloes contribute to food security through production of 67.67 million tons of milk out of a total of 133 million tons (NDDB 2012-2013) and more than 1 million tons of meat. No studies have been reported regarding the association of the AQP7 genes with the semen quality parameters in buffaloes. The present study was undertaken to characterize AQP7 gene and to investigate the association of genetic variants with semen quality in Surti bulls.

MATERIALS AND METHODS

Experimental animals and DNA isolation

Experiment was carried out on 21 Surti bulls maintained at Centralized Semen Collection Centre, Dharwad, Karnataka. The semen samples were collected from April 2014 to March 2015. This Centre is situated at an altitude of 768 meters above the mean sea level on 15.49^{o}N latitude and 74.98^{o}E longitude. This semen station strictly follows the “Minimum Standards for Production of Bovine Frozen Semen” prescribed by Department of Animal Husbandry Dairying and Fisheries, Government of India. Genomic DNA was isolated by high salt method as described by Miller *et al*. (1988) and the working solution was prepared by diluting the stock to 100 ng/µL and stored at -20°C for subsequent analysis. The quality and quantity of DNA were analyzed by agarose gel electrophoresis and UV spectrophotometer. Semen ejaculates were collected by artificial vagina and immediately stored at 37°C in a water bath to evaluate the fresh semen quality traits *viz.* semen volume per ejaculate and sperm concentration. Frozen semen straws of these bulls were collected for estimation of sperm viability and Hypo osmotic swelling (HOS) reaction.

Semen quality analysis

The semen parameters *viz.* Sperm concentration, Semen volume per ejaculate and post thaw motility (PTM) were recorded during April 2014 to March 2015 for each bull from the records maintained in the semen station (SMILE software, IMV technologies, France). These data were divided into winter (November

to February), summer (March to June) and rainy (July to October) season. Sperm viability and HOS reaction in both fresh as well as frozen semen were estimated using standard procedures. To determine the live spermatozoa eosin-nigrosine staining technique was used as described by Bloom (1950) and Hancock (1951). Membrane integrity of spermatozoa was determined by HOS reaction in hypo osmotic solution (150 mOsmol l^{-1}). The HOS reactivity was performed according to the method described by Correa and Zavos (1994).

PCR-SSCP and Sequencing analysis

On the basis of *Bos taurus* AQP7 gene sequence (Ensemble Ref Seq: ENSBTAG00000020105), eight sets of primers were designed using primer3 (http:/www.genome.wi.mit.edu/cgibin/primer/primer-3www.cgi) online software as the sequence for buffalo was not available in the literature. The designed primers were procured from Amnion Biosciences Pvt. Ltd, Bengaluru, India. Details of primer sequences, annealing temperature, exon position and amplicon size used for amplification of all exons of AQP7 gene are presented in Table 1. The Polymerase Chain Reaction (PCR) amplification of DNA Samples was carried out on 50 to 100 ng of genomic DNA in programmed thermo cycler (Genetix, India) containing a final volume of 25 µl of reaction mixture. The PCR reaction mixture consisted of 20 pM of each primer, 200 µM of each dNTPs, 10X Taq Pol assay buffer, 1U Taq polymerase enzyme. The thermo cycler conditions included initial denaturation at 94°C for 5 minutes to exons 1 and 8 whereas 95°C for 10 minutes to rest of the exons. This is followed by 35 cycles of denaturation at 94°C for 1 minute with varying annealing temperatures based on primer set (Figure 1), extension at 72°C for 1 minute followed by a final extension of 72°C for 5 minutes to exons 1 and 8 whereas 10 minutes to rest of the exons. The PCR products were electrophoresed at 100 V in 1.5% agarose gel in 1X TBE buffer containing 0.5 µg/mL ethidium bromide along with a DNA molecular size marker. The gels were visualized and documented using Gel documentation system (Gel doc 1000, Bio-Rad, USA). The PCR products were subjected to SSCP analysis in 10% non-denaturing PAGE (acrylamide and bis-acrylamide ratio, 29:1) gel using 1X TBE buffer in vertical gel electrophoresis unit (Cleaver, UK). The gels were silver stained as described by Sambrook and Russell (2001). SSCP variants were recorded manually based on the number of bands and mobility shifts. Representative samples of each unique PCR-SSCP patterns were custom sequenced using automated ABI DNA Sequencer (Amnion Biosciences Pvt. Ltd., Bengaluru, India) to confirm the mobility shift in each pattern. SNPs were detected by analysis of sequence data using Bio edit Software and Clustal W multiple sequence alignment software by comparing the observed sequence with the bovine AQP7 gene reference sequence (Ensembl Ref Seq: ENSBTAG00000020105).

Statistical analysis

The genotype frequencies in the population were calculated directly by counting the bands appearing in gel. The statistical analysis was carried out using repeated measures of GLM procedure (SAS 9.3). For semen quality parameters like sperm viability and HOS reaction, SSCP pattern and age as fixed factors were fitted in the model (Model 1). According to the age of the bull at the day of sample collection, data were classified into three groups (up to 3 years: 1st age group, 3 to 4 years: 2nd age group and 4 to 5 years: 3rd age group). The data for volume, PTM and concentration were collected

in three different seasons. In addition to the above fixed factors, season was fitted in the model (Model 2). The following models were used to analyze the effect of genotype and non-genetic factors.

$$\text{Model 1: } Y_{ijl} = \mu + G_i + A_j + e_{ijl}$$
$$\text{Model 2: } Y_{ijkl} = \mu + G_i + A_j + S_k + e_{ijkl}$$

Where Y_{ijkl} is observation on the lth bull with i^{th} genotype and j^{th} age in k^{th} season, μ is overall mean, G_i is fixed effect of ith genotype, A_j is fixed effect of jth age (k = 3, 4 and 5 yrs), S_k is fixed effect of kth season of collection (j = 1-winter, 2- summer and 3-rainy), e_{ijkl} is the random error which is NID $(0, \sigma_e^2)$

RESULTS AND DISCUSSION

Entire exonic regions of AQP7 gene along with the exon-intron boundaries (amplicons) were amplified using 8 sets of primers. The PCR-SSCP analysis revealed varying degree of genetic polymorphisms with respect to each of the AQP7 gene exons and amplicons analysed. PCR-SSCP analysis of the exons (1, 4, 5, 7 and 8) and their amplicons showed polymorphism however exons 2, 3 and 6 showed monomorphic pattern in Surti bulls. G→A transition in exon 2 is found among all the bulls under study. The analysis revealed 14 different variants in the different region of AQP7 gene. Exons 1 and 8 (Figure 1) revealed two while exons (amplicons) 4 and 7 showed three and exon 5 revealed four unique SSCP patterns. The frequencies of SSCP variants for each exon in 21 Surti bulls genotyped in the present study are summarised in Table 2.

Representative samples were custom sequenced to confirm the mobility shift in each pattern. Comparison of the observed sequence with the bovine AQP7 gene sequence (Ensembl Ref Seq: ENSBTAG00000020105) using Bio edit software and Clustal W multiple sequence alignment for SNPs detection (Figure 2) and their respective deduced amino acid. A total of 23 SNPs were observed out of which 8 were found in the coding region. The base change, location, and amino acid change in the protein have been summarized in Table 3.

Effect of SSCP variants on semen quality

The analysis of association of SSCP variants on semen quality parameters revealed a significant association of band pattern with Semen quality parameter. SSCP variants of exon 1 had significant ($P \leq 0.05$) effect on HOS reactivity in frozen semen. SSCP variants of exons 5 and 8 had highly significant ($P \leq 0.01$) effect on sperm viability in fresh semen and PTM, respectively. Zhao *et al*. (2009) observed that A264G SNPs located in exon 2 of AQP7 gene is associated with acrosome integrity and sperm viability in Simmental and Charolais bulls. Two SNPs, one located on exon 2 (A264G) and other on exon 3 (G371C) of AQP7 gene in two Turkish breeds using PCR-SSCP technique were reported by Ma *et al.* (2011). Further they reported significant association of A264G with acrosome integrity ($P \leq 0.01$) and motility ($P \leq 0.05$) in frozen semen and G371C with acrosome integrity ($P < 0.05$), percentages of viable sperm ($P \leq 0.05$) and motility ($P \leq 0.01$) in frozen semen. Kumar *et al*. (2014) also reported the same SNPs (A264G, G371C) in Frieswal cattle and observed significant effect of A264G (in exon 2) on motility as well as PTM and G371C (in exon 3) on semen volume, motility and PTM. Both the SNPs were found to influence the quality parameters positively. In Surti bulls SSCP variants of exon 1

Table 1. Primer sequence, location, product size and annealing temperature of the AQP7 gene.

Exon	Primers (5'→ 3')	Primer location	Product size (bp)	T_a (°C)
1	F- GAAGGGGTGCTATTTGGGC R- AGGCAGCAACTCAGGACTAA	601-841	459	58
2	F- AATGAGGGGCCAAGTTCTGT R- AGGGAGTCTTGGTGCATCAG	2008-2056	227	60
3	F- CAAGAGCAGGCATGTGTGG R- AAGGGACAGTGTGATCCAGG	6869-6986	365	61
4	F- AAGGGCAATGCAGGGAGAC R- CTTGTGAAGTGTCTGGGCAG	14763-14886	384	59
5	F- ATCATCGCTGCTGTCATTGC R- AGGACACACTCAGATCTGCC	15252-15389	287	56
6	F- GCAGGTTCACACTCAGTCTG R- TCCAGGGTTCTTGTCCACTC	15597-15715	249	58
7	F- GAGTGGACAAGAACCCTGGA R- CTTTTCCACCCTCCACATGC	15923-16140	413	58
8	F- CATCTTGGCCTGGGGTAGAC R- AACCTGGGAGCTTCTGAGAG	16529-16854	478	56

F = forward, R = reverse, T_a = annealing temperature

Table 2. Frequencies of SSCP variants in exons 1, 4, 5, 7 and 8 of AQP7 gene in Surti bulls.

Exon	Pattern	No of observations	Frequency of SSCP variants
Exon 1	Pattern 1 (P1)	7	0.3333
	Pattern 2 (P2)	14	0.6667
Exon 4	Pattern 1 (P1)	8	0.3810
	Pattern 2 (P2)	6	0.2857
	Pattern 3 (P3)	7	0.3333
Exon 5	Pattern 1 (P1)	5	0.2381
	Pattern 2 (P2)	10	0.4762
	Pattern 3 (P3)	2	0.0952
	Pattern 4 (P4)	4	0.1905
Exon 7	Pattern 1 (P1)	10	0.4762
	Pattern 2 (P2)	9	0.4286
	Pattern 3 (P3)	2	0.0952
Exon 8	Pattern 1 (P1)	16	0.7619
	Pattern 2 (P2)	5	0.2381

Table 3. Summary of SNP observed in AQP7 gene in Surti bulls.

Region	Transversion	Transition	Loci (SNPS)	Amino acid change
Exon-1	NO	T/C	T753C	Pro→Thr
Exon-2	NO	G/A	G2039A	No change
Exon-4	A/C	NO	A14808C	Met→Leu
Exon -5	NO	T/C	T15259C	Tyr→ His
	NO	G/A	G15292A	Ala→Thr
	NO	C/T	C15328T	Arg→cys
	NO	C/T	C15376T	Arg→Trp
Exon-8	T/A	NO	T16788A	-
Total	**2**	**6**	**8**	**6**
Intron-1	C/A	NO	C88OA	
Intron-3	A/T	NO	A14714T	
	NO	G/A	G14730A	
	NO	A/G	A14736G	
	NO	G/A	G7013A	
	C/A	NO	C7015A	
Intron-4	Insertion of G		14901	
	NO	G/A	G14909A	
	NO	C/T	C14917T	
Intron-5	C/A	NO	C15424A	
	C/G	NO	C15427G	
	NO	G/A	G15428A	
Intron-6	NO	C/T	C15866T	
	NO	G/A	G15883A	
	NO	G/A	G15922A	
Total	**5**	**9**	**15**	

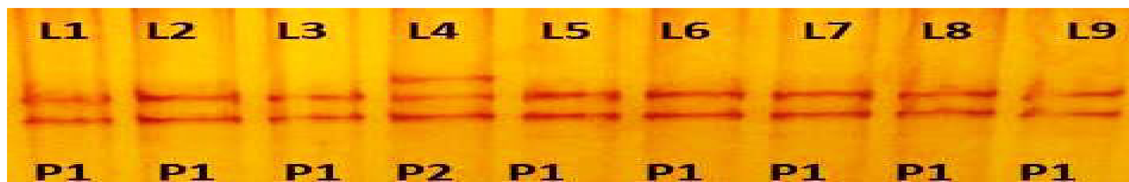

Figure 1. PCR-SSCP patterns in exon 1 of AQP7 gene in Surti bulls.

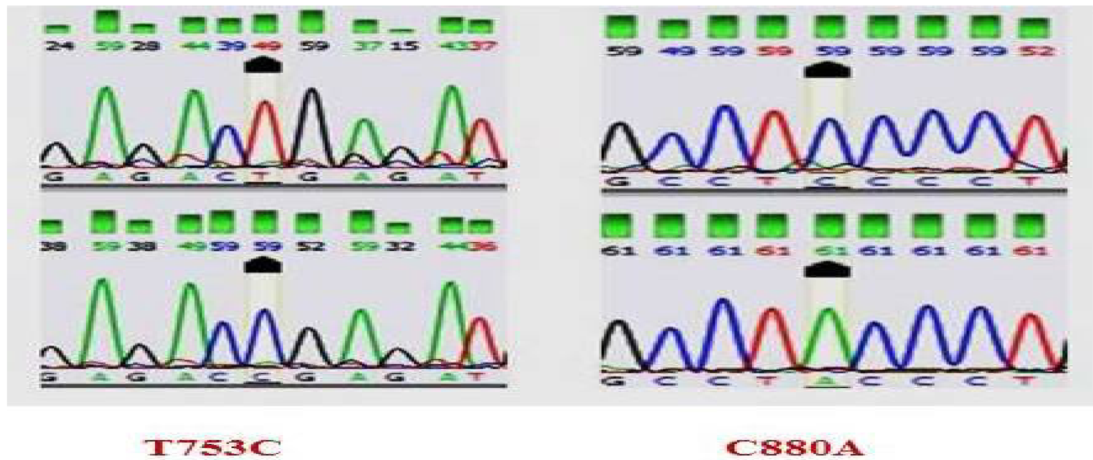

Figure 2. Sanger Trace figures showing sequence comparison of different SSCP variants of buffalo AQP7 gene. The arrow indicates T→C transition at 753 position in exon 1 and C→A transversion at 880 position in intron 1.

had significant (P≤0.05) effect on HOS reactivity in frozen semen. Bulls with SSCP pattern P2 were found to have higher HOS reactivity (57.11±2.66) compared to pattern P1 (46.50±3.99). Least square mean on sperm viability in fresh semen (in percentage) for exon 5 in patterns P1, P2, P3 and P4 were 92.17±1.65, 93.63±1.23, 40.00±3.12 and 91.00±2.21, respectively. Bulls with pattern P3 had lowest mean viability and differed significantly from patterns P1, P2 and P4. Bulls with pattern P2 for exon 8 were found to have higher PTM 46.28±0.88 compared to pattern P1. Current findings revealed that AQP7 gene SNPs may be a convenient marker for selecting good performance bulls with better semen quality parameters in buffaloes.

CONCLUSION

Based on the present result, it is concluded that AQP7 gene mutations affect semen quality traits in buffalo bulls. The results showed association between different SSCP variants and semen traits in three exons. SSCP variants of exon 1 with HOS reactivity in frozen semen, exon 5 with sperm viability in fresh semen and exon 8 with PTM were found to be significantly associated in Surti bulls. These SSCP variants associated with semen parameter offers an opportunity towards identification of markers for semen quality in buffaloes. However, further studies using large number of buffaloes has to be carried out before using them in the Marker Assisted Selection.

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