

SNP Identification in Exon 5 of Aquaporin 7 (AQP7) Gene and their Association with Sperm Quality Traits in Surti Bulls

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ABSTRACT

*The aim of the present study was to study genetic polymorphism in exon 5 of AQP7 gene and to analyze their association with semen quality traits in Surti bulls (n=21). Blood samples were collected from 21 Surti bulls maintained at Centralized Semen Collection Centre, Dharwad, Karnataka and genomic DNA was isolated using high salt method. The quantity of DNA was assessed in a spectrophotometer and quality by gel electrophoresis. The amplicon size of 287 bp covering exon 5 region of AQP7 gene was amplified by polymerase chain reaction (PCR) and the products were subjected to single strand conformation polymorphism (SSCP) analysis. Four unique SSCP banding patterns were revealed by subsequent silver staining of the polyacrylamide gels. The presence of single nucleotide polymorphisms (SNPs) was confirmed by direct sequencing of the PCR products. Sequence analysis revealed 7 SNPs (4 in exonic and 3 in intronic region) as compared to *Bos taurus* (Ensemble Ref Seq: ENSBTAG00000020105). The statistical analysis using repeated general linear model procedure (SAS 9.3) for association study indicated that exon 5 SSCP variants were significantly associated ($P \leq 0.01$) with per cent live sperm in Surti bulls. Bulls with pattern 3 had lowest mean viability and differed significantly from other three patterns. These results suggest that there exists a considerable genetic variation at exon 5 region of AQP7 gene and there is possibility of using this as candidate gene for genetic improvement of buffaloes for sperm quality.*

Key words: AQP7 gene, Surti bulls, PCR-SSCP, SNP, Repeated measures of GLM

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INTRODUCTION

Aquaporins (AQPs) act as plasma membrane channel proteins which participate in water transport driven by osmotic gradients. The fluid movement is strongly associated with the presence of aquaporins (AQPs) in the female and male reproductive systems. AQPs family consists of 13 members (AQP0–AQP12). These are subdivided into: orthodox AQPs, aquaglyceroporins and superaquaporins^{1,2}. Orthodox AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) are water-selective channels and permeable to water but not to small organic and inorganic molecules. AQP11 and AQP12 are included in superaquaporins family. The remaining four AQPs (AQP3, AQP7, AQP9 and AQP10) are included in aquaglyceroporins. These are non-selective water channels, permeable to glycerol, urea and other small non-electrolytes as well as to water^{1,3,4}. Orthodox AQPs and aquaglyceroporins have highly conserved two asparagine-proline-alanine (NPA) boxes that are important for the formation of the water permeating pore. Aquaporin 7 gene is located on chromosome 8 in bovines (BTA 8) and has 7 coding and 1 non-coding exon. It is of 16.25 kilobase pairs. Ishibashi *et al.*⁵ isolated cDNA encoding AQP7, of 269-amino acid protein. AQP7 was first cloned from the testis⁵ and is expressed initially in the round spermatids⁶, is also localized to the sperm tail in rat^{5,6,7}, mouse⁸ and human⁹. Immunohistochemical analysis demonstrated that the AQP7 protein exists in elongated spermatids, testicular spermatozoa, and residual bodies¹⁰. AQP7 was observed in epididymal spermatozoa¹⁰, indicating its possible role in sperm maturation and storage as well as its function in spermatogenesis⁷. AQP7 is permeable to water as well as to urea and glycerol, which is a universally effective cryoprotectant, which suggests its role in the glycerol permeability of sperm during cryopreservation. Single Nucleotide Polymorphisms (SNPs) in the AQP7 gene have been reported to be associated with acrosome integrity, motility, percentages of viable sperm and post thaw motility in cattle^{11,12}. AQP7 is a candidate

antifreeze gene¹¹ expressed in various tissues including male reproductive system¹³. There is no report available on AQP7 gene polymorphisms and sperm quality traits in buffalo. Hence the present study attempted to detect SNPs in the exon 5 regions of the AQP7 gene and to determine their effects on sperm quality traits of fresh and frozen semen in Surti bulls.

MATERIALS AND METHODS

Location and experimental animals

The experimental study was carried out in Surti bulls ($n=21$), maintained at Centralized Semen Collection Centre, Dharwad, Karnataka. The Centre is situated at an altitude of 768 meters above the mean sea level on 15.49°N latitude and 74.98°E longitude. This semen station strictly follows the “Minimum Standards for Production of Bovine Frozen Semen” prescribed by Department of Animal Husbandry Dairying & Fisheries, Govt. of India.

Genotyping of animals

About 10 ml of whole blood was collected from each animal aseptically from Jugular vein into an EDTA coated vacutainer. After collection, the samples were stored at 4°C and DNA was isolated within 24 hours of collection. Genomic DNA was isolated from whole blood by high salt method as described by Miller *et al.*¹⁴. The quality and quantity of DNA were analyzed by agarose gel electrophoresis and UV spectrophotometer. The ratio between OD₂₆₀ and OD₂₈₀ was observed for each sample. DNA sample with a ratio of 1.8 was considered good and taken for further analysis. The working solution was prepared by diluting the stock to 100 ng/μL and stored at -20°C for utilizing as DNA template in PCR. The exon 5 of AQP7 gene was amplified by one set of primer designed from *Bos taurus* AQP7 gene sequence (Ensemble Ref Seq: ENSBTAG00000020105) using primer3 (<http://www.genome.wi.mit.edu/cgi-bin/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. The sequence of primers, their respective nucleotide numbers, targeted region, and amplicon size are given in Table 1. The polymerase chain reaction (PCR) was carried out on about 50 ng/ μ l of genomic DNA in a 25 μ l reaction volume. The PCR reaction mixture consisted of 2.5 μ l of 10x PCR assay buffer containing 1.5 mM MgCl₂, 2.5 μ l of 2.5 mM deoxynucleotides mix (200 μ M of each dNTPs), 0.5 μ l for each of 20 pM/ μ l forward and reverse primers, 1 μ l of 1 U/ μ l *Taq* DNA polymerase and final dilution was made by adding 17 μ l of nuclease free water. Amplification was performed in a programmed thermo cycler (Genetix, India). The thermocycler conditions involved an initial denaturation at 95°C for 10 min followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 56°C, 1 min extension at 72°C and 10 min of final extension at 72°C. PCR program for both the primers was found to be same. The PCR amplified products were checked at 100 V on 1.5% agarose gel in 1X TBE buffer containing 0.5 μ g/mL ethidium bromide along with a DNA molecular size marker (Figure 1). The gels were visualized and documented using Gel documentation system (Gel doc 1000, Bio-Rad, USA). The exon 5 of AQP7 gene PCR products were resolved by PCR-single strand conformation polymorphism (SSCP) technique. Aliquot of 10 μ L PCR products was taken with 10 μ l denaturing solution (95% formamide, 10 mM NaOH, 0.05% Xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA) and mixed properly followed by denaturation at 94°C for 10 min then rapid chilling on ice for 20 min. The mixture was loaded on 10% non-denaturing PAGE (acrylamide and bis-acrylamide ratio, 29:1) gel using 1X TBE buffer for 7 hours (190V) in vertical gel electrophoresis unit (Clever, UK). Band patterns were detected by silver staining as described by Sambrook and Russell¹⁵. SSCP variants were recorded manually based on the number of bands and mobility shifts. Representative PCR products giving unique 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 DNA Baser software, BioEdit software¹⁶ and Clustal W multiple alignments by comparing the observed sequence with the bovine AQP7 gene reference sequence (Ensembl Ref Seq: ENSBTAG00000020105).

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. 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. The sperm concentration was determined by a digital photometer with auto dilutor (SMILE software, IMV Technologies, France). The semen volume was noted directly from sterilized glass conical centrifuge tubes having 0.1 mL calibration which were used for semen collection. These data were collected in winter (November to February), summer (March to June) and rainy (July to October) season. Per cent live and HOST per cent normal spermatozoa were estimated using standard procedures. To determine the percentage of live spermatozoa eosin-nigrosine staining technique as described by Bloom¹⁷ and Hancock¹⁸ was used. Membrane integrity of spermatozoa was determined by HOS reaction in hypo osmotic solution (150 mOsmol l⁻¹). The HOST was performed according to the method described by Correa and Zavos¹⁹. These tests were performed for both fresh and frozen semen samples for all the bulls.

STATISTICAL ANALYSIS

The genotypic frequency in the population was determined by direct counting of the bands appearing in the gels. The association of SSCP variants and effects of non-genetic factors on semen quality parameters was analyzed using repeated measures of GLM procedure (SAS 9.3). For semen quality parameters like per

cent live and HOST, per cent normal spermatozoa (both fresh and frozen), 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 collection of sample, data were classified into three groups like bulls up to 3 years (1st age group), 3-4 years (2nd age group) and 4-5 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 two statistical models (model 1 and model 2) were used to analyze the effect of genotype and non-genetic factors.

Model 1: $Y_{ijk} = \mu + G_i + A_j + e_{ijk}$

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

Where,

Y_{ijk} and Y_{ijkl} = the phenotypic value of traits

μ = overall mean

G_i = fixed effect of genotype (i= pattern 1, 2, 3& 4)

A_j = fixed effect of age group (j = 3, 4 and 5 yrs)

S_k = fixed effect of season of collection (k = 1- winter, 2- summer and 3-rainy)

e_{ijkl} = the random error which is NID (0, σ_e^2).

RESULTS AND DISCUSSION

The exon 5 of AQP7 gene in Surti bulls was found polymorphic by PCR-SSCP analysis and revealed four different conformation patterns on polyacrylamide gel (Figure 2). The frequencies of pattern P1, pattern P2, pattern P3 and pattern P4 in Surti bulls were 0.2381, 0.4762, 0.0952 and 0.1905, respectively. For confirmation of the mobility shift, representative samples of each pattern were custom sequenced. The retrieved sequences representing each of the unique PCR-SSCP band patterns were further analyzed by comparing these sequences with the bovine AQP7 gene sequence (Ensembl Ref Seq: ENSBTAG00000020105) using Clustal-W multiple sequence alignment tool and DNA Baser for detecting Single Nucleotide Polymorphisms and their respective deduced amino acid variations (Figure 3 and 4). The observed SNPs in exon 5 of AQP7 gene is presented in Table 2. There were 7 nucleotide changes in the amplified regions. Out of these, four SNPs were found in exonic region and

three were in intronic region. Analysis of identified SNPs of exonic region of AQP7 gene by using ExPASy translate tool revealed amino acid changes for all the four SNPs.

ASSOCIATION OF GENETIC VARIANTS WITH SEMEN QUALITY

The analysis of association of different band pattern on semen quality parameters revealed a significant association of band pattern with per cent live sperm (Table 3) in fresh semen and no effect on sperm concentration, volume per ejaculate, PTM and Hypo Osmotic Swelling Test (HOST). SSCP variants of exon 5 was found to have significant ($P \leq 0.01$) effect on sperm viability in fresh semen in Surti bulls. The association of AQP7 gene with semen parameters in Cattle were reported by various researchers. Using PCR-SSCP technique, Zhao et al.²⁰ reported a SNP (A→G) in exon 2 of AQP7 gene and its association with semen quality in Simmental and Charolais bulls. Ma et al.¹¹ reported two SNPs one located on exon 2 (A264G) and other on exon 3 (G371C) of AQP7 gene in two Turkish breeds, Simmental and Charolais bulls. A264G transition had significant association with acrosome integrity ($P \leq 0.01$) and motility ($P \leq 0.05$) in frozen semen and G371C transversion with acrosome integrity ($P \leq 0.05$), percentages of viable sperm ($P \leq 0.05$) and motility ($P \leq 0.01$) in frozen semen. Same SNPs (A264G, G371C) were reported by Kumar et al.¹² in Frieswal cattle. They observed significant effect of A264G (in exon 2) on motility as well as post thaw motility and G371C (in exon 3) on semen volume, motility, and post thaw motility. Both these SNPs were found to influence the quality parameters positively. No report is available on the association of genetic variants of AQP7 gene on semen quality traits in buffaloes to compare the above findings. The least squares mean of percent live sperm with respect to pattern P1, P2, P3 and P4 were 92.17 ± 1.65 , 93.63 ± 1.23 , 40.00 ± 3.12 and 91.00 ± 2.21 , respectively. The results indicated that the bulls with SSCP pattern P3 had lower viability than the other three SSCP patterns. The current findings indicated that there is high variability

in exon 5 of AQP7 gene and genetic variants are associated with semen parameters in buffalo. These SNPs may be a convenient

marker for selecting good performance bulls with better semen quality parameters in buffaloes.

Table 1: Primer sequences used to amplify AQP7 gene

Gene	Forward and reverse sequence (5' to 3')	No. of base	Targeted region on gene	Expected product size	Annealing temp (°C)
AQP7 (Exon5)	F-ATCATCGCTGCTGTCATTGC R-AGGACACACTCAGATCTGCC	20 20	15181 - 15467	287 bp	56

Table 2: SNPs observed in amplicon covering exon 5 and intron 5 of AQP7 gene in Surti bulls

Region	Transversion	Transition	Loci (SNPS)	Amino Acid Change
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
Total	0	4	4	4
Intron-5	C/A	NO	C15424A	
	C/G	NO	C15427G	
	NO	G/A	G15428A	
Total	2	1	3	

Table 3: Semen quality parameters (Mean ± SE) with respect to different SSCP band patterns of exon 5 of AQP7 gene

Semen quality parameters	Exon5 of AQP7 gene				P-Value	
	Pattern 1	Pattern 2	Pattern 3	Pattern 4		
Sperm Concentration (million cells/ml)	701.85±90.31	904.61±61.54	785.69±125.42	771.00±102.40	0.31	
Volume/ejaculate(ml)	3.05±0.33	2.92±0.22	3.04±0.46	3.27±0.37	0.87	
% live spermatozoa	Fresh	92.17 ^a ±1.65	93.63 ^a ±1.23	40.00 ^b ±3.12	91.00 ^a ±2.21	0.01**
	Frozen	69.67±4.01	68.50±3.00	68.15±2.99	75.33±4.39	0.36
HOST positive (%)	Fresh	75.33±4.86	63.25±3.64	45.00±9.22	62.50±6.52	0.16
	Frozen	62.50±4.83	49.50±3.62	55.05 ±3.52	51.50±6.48	0.24
Post thaw motility (%)	46.80±2.14	45.30±1.46	45.64±2.97	44.42±2.43	0.88	

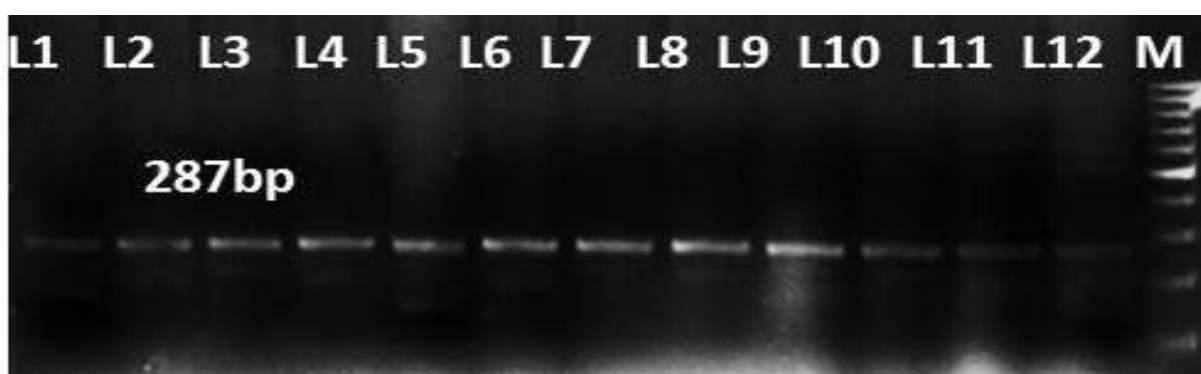


Fig. 1: PCR amplification of exon 5 of AQP7 gene in Surti bull (L 1-12= 287bp; M=100bp)

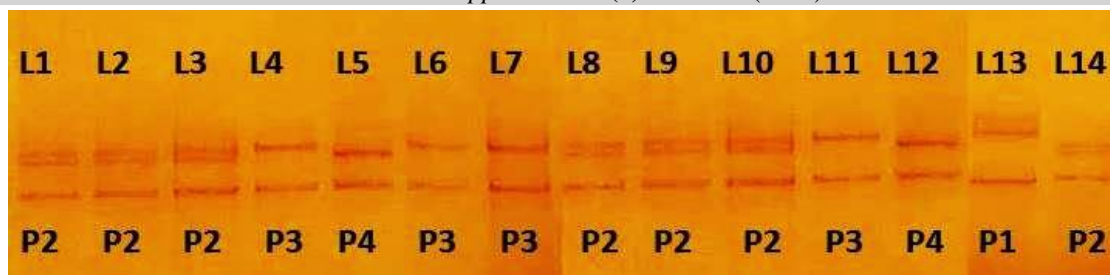


Fig. 2: Different SSCP band patterns observed in exon 5 of AQP7 gene

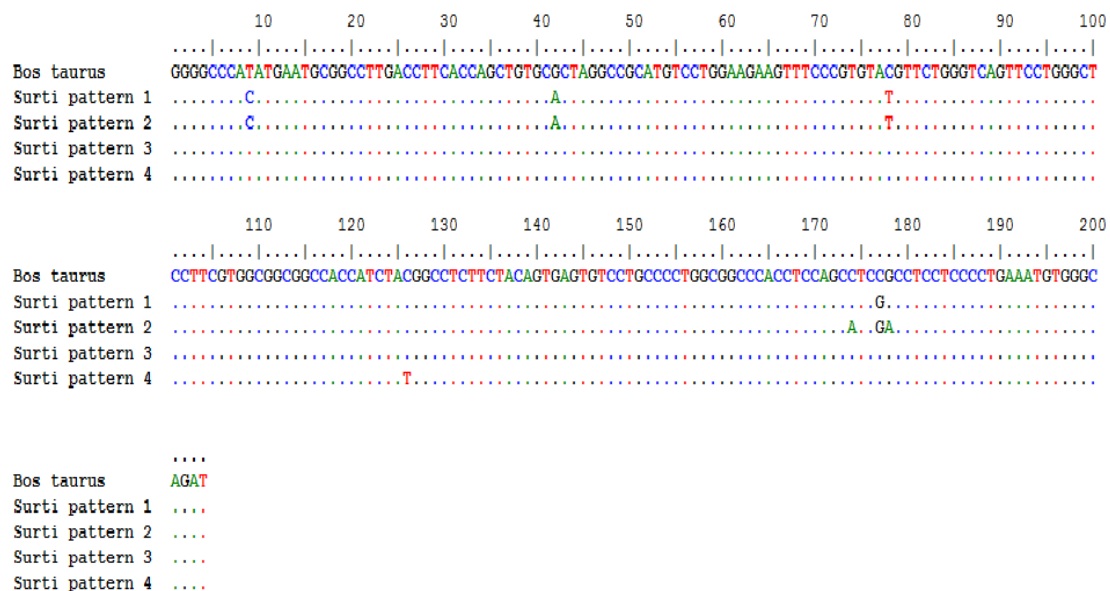


Fig. 3: CLUSTAL W Multiple sequence Alignment of exon 5 (AQP7 gene) showing 7 SNPs in Surti bulls. Reference sequence (Ensembl Ref Seq: ENSBTAG00000020105)

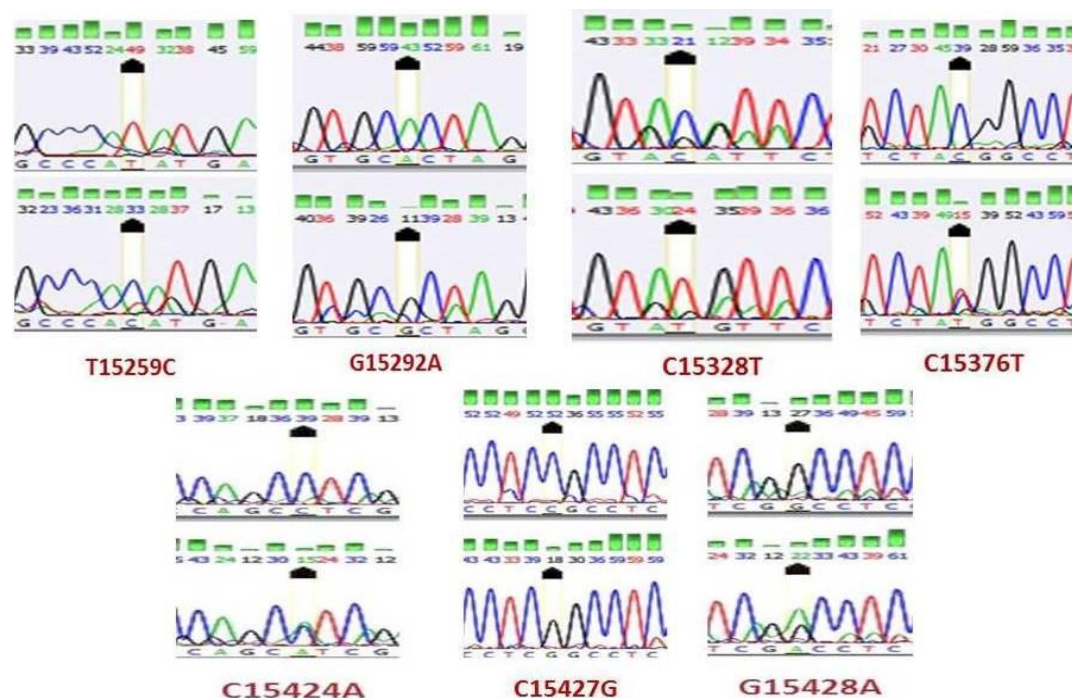


Fig. 4: Sanger Trace figure of SSCP variant sites of exon 5 in Surti bulls

CONCLUSIONS

The study was carried out in Surti bulls ($n=21$) with the objectives to identify genetic polymorphism in the targeted regions (exon 5) of AQP7 gene and to analyze their association with semen quality traits. On the basis of comparative sequence analysis 7 SNPs (4 in exonic and 3 in intronic region) were detected. Four different SSCP band patterns were revealed by subsequent silver staining. SSCP variants in exon 5 were significantly associated with per cent live sperm in Surti bulls. Bulls with SSCP pattern P3 had lower viability than the other three SSCP patterns. The genetic variants observed in exon 5 of AQP7 and their genetic association with sperm quality traits indicated the possibilities of using SSCP patterns as marker for semen quality parameters in buffaloes. However, further studies using large number of animals need to be carried out to validate the marker data before using them in the Marker Assisted Selection (MAS).

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