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ONLINE RESOURCES

SNPs at exonic region of *aquaporin-7* (*AQP7*) gene may affect semen quality parameters among crossbred bulls

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[Kumar S., Deb R., Singh U., Ganguly I., Mandal D. K., Singh R., Sharma S., Sengar G., Singh R., Kumar M. and Sharma A. 2014 SNPs at exonic region of *aquaporin-7* (*AQP7*) gene may affect semen quality parameters among crossbred bulls. *J. Genet.* **93**, e108–e112. Online only: http://www.ias.ac.in/jgenet/OnlineResources/93/e108.pdf]

Introduction

Selection of high fertility crossbred bulls is a prerequisite for the cost-effective frozen semen production and dissemination of superior germplasm. Promoting faster genetic improvement in sire herd and quality parameters of bull semen have tremendous importance, as the success of artificial insemination depends to a great extent on the good semen quality which in turn are affected by environment, management, physiological status and genetic factors (Mathevon *et al.* 1998). Many studies have been carried out to disclose the genetic basis of semen quality traits in different farms and laboratory animals (Huhtaniemi and Alevizaki 2007; Druet *et al.* 2009; Xing *et al.* 2009; Wang *et al.* 2011; Deb *et al.* 2013; Ganguly *et al.* 2013). Due to their low heritability, the direct selection of semen quality traits is challenging (Mathevon *et al.* 1998). However, the candidate genes having major effect on semen quality traits may be beneficial as a marker towards selection of semen quality traits.

Aquaporins (AQPs) were first detected as plasma membrane channel proteins participating in water transport driven by osmotic gradients. There are totally 13 members in the AQP family (AQP0–AQP12), the expression of one or more of these is ubiquitous in mammalian cells (Ishibashi *et al.* 2009). Among them, four (AQP3, AQP7, AQP9 and AQP10) are 'aquaglyceroporins', that allow passage of glycerol, two (AQP11 and AQP12) are 'superaquaporins' and the remaining seven are largely water-selective AQPs. It is well reported that the germ cells express *AQP7* both at mRNA as well as at protein levels. *AQP7* was first cloned from the testis (Ishibashi *et al.* 1997) and is expressed initially in the round spermatids (Kageyama *et al.* 2001), is also localized to the sperm tail in rat (Ishibashi *et al.* 1997; Suzuki-Toyota *et al.* 1999; Kageyama *et al.* 2001), mouse (Skowronski *et al.* 2007) and human (Saito *et al.* 2004). The plausible role of AQP7 in male reproductive system is unclear as *AQP7* gene knockout mice are fertile producing normal functional spermatozoa (Sohara *et al.* 2007), whereas an association with sperm motility has been suggested in infertile men (Saito *et al.* 2004). Based on the reality that *AQP7* is a candidate antifreeze gene expressed in various tissues including male reproductive system (Moretti *et al.* 2011), this study was designed to investigate the association between the SNPs located in bovine *AQP7* gene with certain semen quality parameters such as semen volume, concentration, motility, post-thaw motility (PTM), viability and hypoosmotic swelling (HOS) response of Frieswal (HF × Sahiwal) bull semen.

Scanty of reports are available till now, whether the genetic variation of *AQP7* effects the semen quality parameters of cattle, except for a single report by Ma *et al.* (2011), who observed that A264G and G371C SNPs located in *AQP7* gene are associated with semen motility among two Turkish breeds. The present study is further extension of that earlier work to reevaluate the association of *AQP7* genetic variation (at two SNPs i.e. A264G at exon 2 and G371C at exon 3) on certain semen quality traits (mentioned earlier) among Frieswal (HF × Sahiwal) crossbred bulls developed in India. This study also aimed to associate the effect of combined SNPs on the semen quality traits of Frieswal bulls.

Material and methods

A total of 96 mature Frieswal bulls were included in this study. Semen was collected using artificial vagina from each bull during the period from December 2010 to November 2011. Numbers of ejaculates collected per bull varied from

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Keywords. AQP7; Frieswal cattle; semen quality.

50–70 per ejaculate. The data collection of semen quality parameters accurately followed the previous work of our laboratory (Deb *et al.* 2013). The ejaculates after collection were immediately stored at 37°C in a water bath to evaluate the fresh semen quality traits including semen volume per ejaculate (mL), sperm motility (%), viability (%) and sperm concentration (M/mL). The fresh semen was then diluted with glycerol-egg yolk-citrate, processed and cryopreserved. After storing in liquid nitrogen for 1–2 days, two straws were randomly obtained from each ejaculate and thawed at 37°C for 60 s and immediately evaluated for PTM (%) with light microscopy. To measure the sperm plasma membrane integrity, hypo-osmotic swelling test (HOST) was performed as described by Ganguly *et al.* (2013). Genomic DNA was extracted from the sperm using GenElute^{TM}Blood Genomic DNA Kit (Sigma-Aldrich, St Louis, USA). DNA samples were dissolved in elution buffer (supplied with kit) and stored at −20°C for future use.

The primers used in this experiment are based on earlier report by Ma *et al.* (2011). The amplification of the semen DNA samples was carried out in a 25 μL reaction volume containing 50 ng/μL of template DNA, 1× buffer (Tris-HCl 100 mmol/L, pH 8.3; KCl 500 mmol/L), 0.25 μmol/L primers, 2.0 mmol/L $MgCl_2$, 0.25 mmol/L dNTPs and 0.5 U *Taq* DNA polymerase (Sigma-Aldrich). The polymerase chain reaction (PCR) protocol was 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 55°C (A264G) and 58°C (G371C) for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products were separated on 1.0% agarose gel (Sigma-Aldrich) including 0.5 μg/mL of ethidium bromide, photographed under Gel Documentation system (Alpha imager, San Leandro, USA). Amplified PCR products were gel purified and outsourced sequencing. PCR product, 5 μL, was digested by 10 U *Taa*I (New England Biolabs, Ipswich, USA) at 65°C for 12 h. Fragments were detected by running the digested products in 2.5% agarose gel electrophoresis and documented using Gel Documentation system (Alpha Imager). Different genotypes obtained for both the loci were subjected to TA cloning using pTZ57R plasmid (InsTA clone PCR Cloning kit, Fermentas, Fisher Scientific, Pittsburgh, USA. The positive clones were confirmed by restriction enzyme analysis. Positive clones from each genotype were subjected to DNA sequencing.

Genotype frequencies of both SNPs and their combinations were calculated by direct counting. Data pertinent to semen quality parameters including volume, sperm concentration, motility, PTM, viability and HOS reactivity of different genotypes were subjected to analysis of variance (ANOVA) using the general linear model (GLM) applying SPSS (Statistical Package for Social 89 Sciences) for SAS software (Statistical Analysis System 9.1, SAS Institute Inc.), computer software programs according to the following statistical model:

$y_{ik} = \mu + G_i + A_k + e_{ik}$, where y_{ik} is phenotypic value of sperm quality traits; μ is the population mean, G_i is fixed effect of genotypes, A_k is fixed effect of age, e_{ik} is random residual error.

Results and discussion

In this study two SNPs (A264G located at exon 2; G371C located at exon 3 regions) located in bovine *AQP7* gene (chr. 8: 79,256,000–79,294,000) were targeted based on study reported earlier by Ma *et al.* (2011), in Simmental and Charolais bulls maintained in Turkey. Bulls were classified as good and poor category according to their seminal progressive motility. Motility more than 50% was considered as good category and less than that was considered as poor category. Seminal attributes of the good and poor semen producer Frieswal bulls used in the present study are shown in our earlier report (Ganguly *et al.* 2013) (table 1).

PCR-RFLP was used to identify genotypic pattern of both the SNPs among Frieswal bulls. The results confirmed the presence of a single homozygous genotype (AA and CC for A264G and G371C locus, respectively) and heterozygous genotypes (AB and CD for A264G and G371C loci, respectively). The 221 bp comprising amplicon of the homozygous AA allele of A264G locus is digested by *Taa*I resulting in the diagnostic fragments of 129 and 92 bp and thus the heterozygous allele (AB) having 221, 129 and 92 bp fragments (figure 1). Further, the genotypic frequency of AB (0.39) was lower than AA (0.66) in Frieswal bulls (table 2). No homozygous BB genotypes were present among the studied population. This result supports the earlier findings by Ma *et al.* (2011), who observed that the frequency of AA was greater than that of AB in Simmental and Charolais bulls. Similarly, in polymorphic locus G371C, CC genotype having 199 bp and CD genotype having 199, 129 and 70 bp fragments (figure 1) with genotype frequency of 0.89 and 0.10 respectively (table 3). No homozygous DD genotypes were present so far. Sequencing of all the four different clones (each genotype from two SNPs) revealed genotypic variability (AA and AB for A264G; CC and CD for G371C SNP locus) of *AQP7* gene among Frieswal bull semen (figure 2). The results here again support the earlier findings by Ma *et al.* (2011).

Table 1. Association of A264G SNP of *AQP7* gene with semen quality parameters (mean ± SD) of Frieswal bulls.

Genotype	Genotype frequency	Volume (mL)	Concentration (M/mL)	Motility (%)	Viability (%)	HOS (%)	PTM (%)
1 AA ($n = 66$)	0.69	4.26 ± 0.22^{ns}	1461.15 ± 106.23^{ns}	47.05 ± 0.49^{a}	64.36 ± 1.24^{ns}	55.52 ± 6.23^{ns}	30.38 ± 0.47^{c}
2 AB ($n = 30$)	0.31	4.86 ± 0.66^{ns}	937.22 ± 88.67^{ns}	40.55 ± 0.42^{b}	66.82 ± 0.88^{ns}	62.03 ± 8.44^{ns}	37.29 ± 0.53^{d}

Different lower case subscript letters of least squares mean within a row mean significant difference at $P < 0.05$; ^{ns}nonsignificant.

A possible interpretation of the lack of homozygous alleles for one allele might be that animals with that genotype are not viable.

Each sequence after alignment was deposited to Bankit, NCBI for accession numbers (KF765574 and KF765575 for AA and AB allele of A264G locus; KF765576 and KF765577 for CC and CD allele of G371C locus). Thus our findings demonstrated that genetic polymorphism of *AQP7* gene exists among Frieswal crossbred bulls developed in India. Among the two SNPs chosen, A264G substitution was present two nucleotides ahead of ATG start codon which may indicate that point mutations may influence the transcription process (Mayo *et al.* 2006). Changing the GC content at translation initiation site supposed to alter the ribosome binding efficiency at ATG starting point and thus finally change the AQP7 protein expression. Again, at SNP G371C, substitution causes E to Q amino acid changes. While both the amino acid substitutions are hydrophilic in nature, they may not change the water channel structure of AQP7, which indicates that the amino acids in AQP7 protein are relatively conserved (Ma *et al.* 2011).

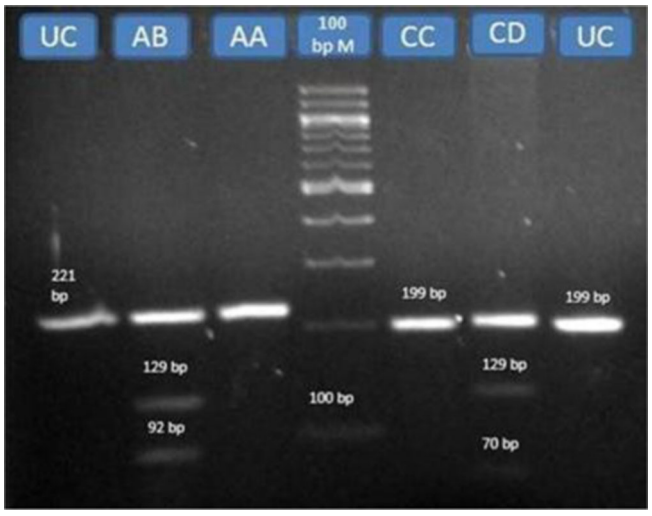

Figure 1. PCR-RFLP patterns of A264G and G371C SNPs located in *AQP7* gene of Frieswal bulls. UC, uncut; AB and AA, genotypes of A264G; CC and CD, genotypes of G371C.

The association studies performed for the identified genotypes of both the SNPs (A264G and G371C) with certain semen quality parameters (semen volume, concentration, motility, PTM, viability and HOS reactivity) of studied Frieswal bulls. The results revealed that in polymorphic locus A264G, the AA genotypes had significantly ($P < 0.05$) higher motility (47.05±0.49, $P = 0.0261$) and PTM (30.38± 0.47, $P = 0.0447$) than AB genotypes (table 1). Again, in locus G371C, the results revealed that CC genotypes had significantly higher semen volume (4.68 ± 0.24, $P = 0.0343$), motility (46.11 ± 0.29, $P = 0.0487$) and PTM (39.89 ± 0.23, $P = 0.0494$) than CD genotype bulls. However, we could not get any association against semen concentration, PTM and HOS reactivity (table 2).

It is well known that genotype of an individual SNP may have effects on other SNPs and thus the genotype combination effect is the indication of interaction between multiple SNPs (Zheng *et al.* 2011). Studying the inheritance of combined genotypes is often more fruitful than focussing on a single genotype (Fallin *et al.* 2001). In this study, we identified four combined genotypes (AACC, ABCC, AACD and ABCD) of two SNPs combination (A264G and G371C) in the studied cattle populations. Among the four combinations, the ABCD genotype frequency was very low and thus it was excluded from the association. Association analysis revealed that, AACC genotype had significantly higher semen volume (4.56 ± 0.29, $P = 0.0234$) and highest fresh semen motility

Table 2. Association of G371C SNP of *AQP7* gene with semen quality parameters (mean ± SD) of Frieswal bulls.

	Genotype	Genotype frequency	Volume (mL)	Concentration (M/mL)	Motility (%)	Viability (%)	HOS (%)	PTM (%)
1	CC ($n = 86$)	0.89	4.68 ± 0.24^{a}	1009.19 ± 109.41^{ns}	46.11 ± 0.29^{c}	66.43 ± 1.43^{ns}	59.73 ± 0.82^{ns}	39.89 ± 0.23^{e}
2	CD ($n = 10$)	0.10	3.40 ± 0.21^{b}	1081.26 ± 72.21^{ns}	39.17 ± 0.32^{d}	65.28 ± 1.32^{ns}	57.15 ± 0.74^{ns}	31.27 ± 0.28^{f}

Different lower case superscript letters of least squares mean within a row mean significant difference at $P < 0.05$; ^{ns}nonsignificant.

Table 3. Association of combined A264G and G371C SNPs of *AQP7* gene with semen quality parameters (mean ± SD) of Frieswal bulls.

	Genotype	Volume (mL)	Concentration (M/mL)	Motility (%)	Viability (%)	HOS (%)	PTM (%)
1	AACC ($n = 58$)	4.56 ± 0.29^{a}	978.23 ± 105.51^{ns}	46.24 ± 0.21^{c}	68.26 ± 1.21^{ns}	57.23 ± 0.88^{ns}	38.27 ± 0.22^{e}
2	AACD ($n = 7$)	3.25 ± 0.27^{b}	1062.34 ± 82.21^{ns}	37.09 ± 0.29^{d}	64.48 ± 1.29^{ns}	56.99 ± 0.84^{ns}	32.34 ± 0.29^{f}
3	ABCC ($n = 30$)	3.91 ± 0.23^{a}	998.67 ± 94.45^{ns}	41.23 ± 0.22^{c}	66.66 ± 0.89^{ns}	56.67 ± 0.82^{ns}	36.67 ± 0.23^{e}

Different lower case superscript letters of least squares mean within a row mean significant difference at $P < 0.05$; ^{ns}nonsignificant.

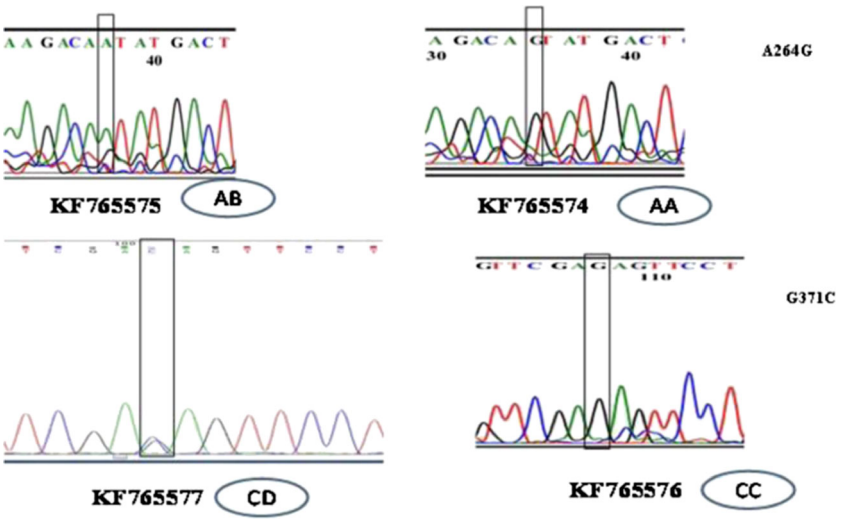

Figure 2. Chromatograph and accession number obtained for different genotype of bovine *AQP7* gene among Frieswal bull semen.

(46.24. ± 0.21, $P = 0.0378$) and PTM (38.27 ± 0.22, $n = 0.0462$) percentage than ABCC and AACD genotypes (table 3).

Results from the association analysis provided preliminary evidence that the bovine *AQP7* gene could be used as a candidate gene or molecular marker for selecting good semen quality traits among crossbred bulls. Further study is needed for investigating the role of other aquaporin gene families for their relationship with bull semen quality parameters.

Acknowledgement

This study was supported by World Bank funded National Agricultural Innovation Project, Indian Council of Agricultural Research, New Delhi, India.

References

Deb R., Kumar S., Singh U., Tyagi S., Mandal D. K., Sengar G. *et al.* 2013 Evaluation of three bovine Y specific microsatellite loci as an alternative biomarkers for semen quality traits in crossbred bull. *Anim. Reprod. Sci.* **142**, 121–125.

Druet T., Fritz S., Sellem E., Basso B., Gérard O., Salas-Cortes L. *et al.* 2009 Estimation of geneticparameters and genome scan for 15 semen characteristics traits of Holstein bulls. *J. Anim. Breed. Genet.* **126**, 269–277.

Fallin D., Cohen A., Essioux L., Chumakov I., Blumenfeld M., Cohen D. and Schork N. J. 2001 Genetic analysis of case/control data using estimated haplotype frequencies: application to APOE locus variation and Alzheimer's disease. *Genome Res.* **11**, 143–151.

Ganguly I., Gaur G. K., Kumar S., Mandal D. K., Kumar M., Singh U. *et al.* 2013 Differential expression of protamine 1 and 2 genes in mature spermatozoa of normal and motility impaired semen producing crossbred Frieswal (HF × Sahiwal) bulls. *Res. Vet. Sci.* **94**, 256–262.

Huhtaniemi I. and Alevizaki M. 2007 Mutations along the hypothalamic-pituitary–gonadal axis affecting male reproduction. *Reprod. Biomed. Online* **15**, 622–632.

Ishibashi K., Kuwahara M., Gu Y., Kageyama Y., Tohsaka A., Suzuki F. *et al.* 1997 Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J. Biol. Chem.* **272**, 20782–20786.

Ishibashi K., Hara S. and Kondo S. 2009 Aquaporin water channels in mammals. *Clin. Exp. Nephrol.* **13**, 107–117.

Kageyama Y., Ishibashi K., Hayashi T., Xia G., Sasaki S. and Kihara K. 2001 Expression of aquaporins 7 and 8 in the developing rat testis. *Andrologia* **33**, 165–169.

Ma T. H., Liu J. F., Zhao R. F., Jiang H., Dai L. S., Zhao Y. M. *et al.* 2011 Association analysis of aquaporin 7 (AQP7) gene variants with semen quality and fertility in bulls. *Turk. J. Vet. Anim. Sci.* **35**, 63–66.

Mathevon M., Buhr M. M. and Dekkers J. C. 1998 Environmental, management, and genetic factors affecting semen production in Holstein bulls. *J. Dairy Sci.* **81**, 3321–3330.

Mayo A. E., Setty Y., Shavit S., Zaslaver A. and Alon U. 2006 Plasticity of the cis-regulatory input function of a gene. *PLoS Biol.* **4**, e45.

Moretti E., Geminiani M., Terzuoli G., Renieri T., Pascarelli N. and Collodel G. 2011 Two cases of sperm immotility: a mosaic of flagellar alterations related to dysplasia of the fibrous sheath and abnormalities of head-neck attachment. *Fertil. Steril.* **95** 1787, e19–e23.

Saito K., Kageyama Y., Okada Y., Kawakami S., Kihara K., Ishibashi K. *et al.* 2004 Localization of aquaporin-7 in human testisand ejaculated sperm: possible involvement in maintenance of sperm quality. *J. Urol.* **172**, 2073–2076.

Skowronski M. T., Lebeck J., Rojek A., Praetorius J., Fuchtbauer E. M., Frokiaer J. and Nielsen S. 2007 AQP7 is localized in capillaries of adipose tissue, cardiac and striated muscle: implications in glycerol metabolism. *Am. J. Physiol. Renal Physiol.* **292**, 956–965.

Sohara E., Ueda O., Tachibe T., Hani T., Jishage K., Rai T. *et al.* 2007 Morphologic and functional analysis of sperm and testes in Aquaporin 7 knockout mice. *Fertil. Steril.* **87**, 671–676.

Suzuki-Toyota F., Ishibashi K. and Yuasa S. 1999 Immuno histochemical localization of a water channel, aquaporin 7 (AQP7), in the rat testis. *Cell Tissue Res.* **295**, 279–285.

Wang L. B., Fan J. S., Yu M. J., Zheng S. Y. and Zhao Y. J. 2011 Association of goat (Capra hircus) CD4 geneexon 6 polymorphisms with ability ofsperm internalizing exogenous DNA. *Mol. Biol. Rep.* **38**, 1621–1628.

Xing Y., Ren J., Ren D. and Guo Y. 2009 A whole genome scanning for quantitative trait loci on traits related to sperm quality and ejaculation in pigs. *Anim. Reprod. Sci.* **114**, 210–218.

Zheng X., Ju Z., Wang J., Li Q., Huang J., Zhang A. *et al.* 2011 Single nucleotide polymorphisms, haplotypes and combined genotypes of LAP3 gene in bovine and their association with milk production traits. *Mol. Biol. Rep.* **38**, 4053–4061.

Received 24 January 2014; accepted 9 June 2014,
Unedited version published online: 26 June 2014
Final version published online: 13 November 2014