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Elucidating the role of bovine GnRHR on semen quality traits among crossbred bull

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ABSTRACT

Gonadotropin-releasing hormone receptor (GnRHR) gene plays an important role in endocrine regulation as well as gonadal development. Two SNPs (G286A and T340C) of GnRHR were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and 3 combinations of these SNPs were observed. The single SNP and their genetic effects on semen quality traits were evaluated and a significant association with semen volume and concentration was found in T340C SNP. The mean semen volume and concentration of individuals with genotype TT showed significantly higher sperm concentration and lower semen volume compared to bulls with heterozygote TC genotype. The results of combined genotypes analysis of two SNPs showed that GGTC had the significantly highest semen volume and lowest concentration compare to the others.

Key words: Frieswal, GnRHR, G286A, Semen, T340C

Numerous studies have been conducted to expose the genetic basis of semen quality traits among different farm as well as laboratory animal species (Deb *et al.* 2013; Ganguly *et al.* 2013).

Gonadotropin-releasing hormone receptor (GnRHR) plays an important role in male reproductive performance (Albertson *et al.* 2008). GnRH and GnRHR thus play a critical role in coordinating sexual differentiation and mammalian reproduction. GnRHR may therefore be a potential marker for semen quality traits. It has been observed that genetic variations within GnRHR contribute to the regulation of pubertal timing in human, mouse and bovine (Ferreira *et al.* 2010, Milazzotto *et al.* 2008), semen quality traits in boar (Lin *et al.* 2006) and bull (Yang *et al.* 2011). Owing to the importance of GnRHR as biomarker for the assessment of semen quality traits, this study attempts to characterize 2 SNPs (G286A and T340C) at exon 1 region of bovine GnRHR gene among Frieswal (HF × Sahiwal) bulls.

MATERIALS AND METHODS

Experimental animals and data collection: Mature Frieswal bulls (96) were included in this study. Semen was collected using an artificial vagina (42–45°C) from each bull during the period from December 2010 to Nov 2011 at early morning. Number of ejaculates collected per bull were varied from 40–70. As per Kumar *et al.* (2015), immediately after collection, the ejaculates were stored at 34°C in a water bath to evaluate the fresh semen quality traits including semen volume per ejaculate (Vol, ml), sperm motility (MOT, %) and sperm concentration (SCON, in M/ ml). The concentration of sperm was measured by using photometer readings. The fresh semen was then diluted with glycerol-egg yolk-citrate extender, processed and cryo-preserved. After storage in liquid nitrogen for 1–2 days, 2 straws were randomly obtained from each ejaculate and thawed at 37°C for 60 sec, and immediately evaluated for post thaw motility (PTM, %) with light microscopy. Genomic DNA was extracted from the sperm using blood genomic DNA kit. The DNA samples were dissolved in elution buffer (supplied with kit) and stored at –20°C for future use. Both eosin nigrosin (EN) vital stain and hypo-osmotic swelling test (HOST) were included for the evaluation of spermatozoal integrity. EN test were performed after preparation of smears by mixing 20 µl of sperm fraction and an equal amount of EN solution (1 g eosin, 5 g nigrosin, 2.9 g sodium citrate in 100 ml of distilled water). Percentage of membrane-intact sperm examined by EN was detected by counting around 200 sperm under magnification (400×) with bright-field microscopy. Again, the response of bovine spermatozoa to the hypoosmotic swelling test was measured with fructose and sodium citrate solution (150 mOsm/L).

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100 µl of raw semen was supplemented to 1.0 mL of pre-warmed (37°C) HOS solution and incubated for 1 h at 37°C. Likewise, for each ejaculate a control sample was incubated in 2.9% sodium citrate solution. Followed by incubation, a drop of sample was placed on a clean grease free glass slide, covered with a cover slip and examined under phase contrast microscopy at 400× magnification.

PCR conditions: Two reported SNPs of G286A (GenBank no:GU222218) and T340C (GenBank no: GU222219) in the exon I region of the bovine GnRHR gene were genotyped in all the 96 Frieswal bulls using *Mbo*II (GAAGA) and *Bsp*MI (ACCTGC) restriction endonuclease, respectively. Two pair of published primers (Pair 1: F-5'-TATCAGAAGTGCCAGAAACACGAGTC-3' and R-5'-GCTCTCCAGCATACCATTGAACAGT-3'; Pair 2: F-5'-GAAACTTCAGAATTGGACTCAA AGG-3' and R- 5'-GCTCTCCA GCATACCATTGAACAGT-3') were synthesized to amplify 384 and 161 bp amplicons encompassing G286A and T340C, respectively, (Yang *et al*. 2011). The PCR was performed in a 25 µl reaction mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 10 pmol of each primers, 1U Taq DNA polymerase and 50 ng of genomic DNA as template. The cycling protocol was initial denaturation for 5 min at 95°C followed by 35 cycles (94°C for 50 sec, 61°C annealing for polymorphic locus T340C and 62°C for polymorphic locus G286A for 30 sec, 72°C for 45 sec), with a final extension at 72°C for 10 min.

Genetic screening: Polymorphisms at G286A and T340C were detected by PCR-RFLP. For this purpose, PCR products were digested with *Mbo*II and *Bsp*MI respectively. The digestion mixture contained 6 µl PCR products, 1X digestion buffer and 6.0 U of each enzyme. Digestion was carried for overnight at 37°C. Fragments were detected by running the digested products in 2.5% agarose gel electrophoresis and documented by using Gel Documentation system.

Statistical analysis: Genotype and gene frequencies for both the SNPs (G286A and T340C) and their combinations were calculated by direct counting. Associations of SNP genotypes and semen quality traits including volume, sperm concentration, IPM, PTM, viability and HOST were analyzed by SPSS for Window version 11.0.1 SPSS Inc. USA computer software. Only factors that affected the records were fitted in the final statistical model shown as following:

$$y_{ij} = \mu + G_i + e_{ij}$$

where, y_{ij} is phenotypic value of sperm quality traits; µ is the population mean; G_i is fixed effect of genotypes; e_{ij} is random residual error.

RESULTS AND DISCUSSION

In the present study, polymorphisms at exon 1 region of GnRHR gene were detected by PCR-RFLP. Two mutations (G286A and T340C), recognized by endonucleases *Mbo*II and *Bsp*MI, respectively, were screened among the bull population. *Mbo*II digestion of amplified products showed three fragments (170, 118 and 96 bp) for GG genotype and four fragments (214, 170, 118 and 96 bp) for GA genotype (Figs 1 A and 1B). However, no AA genotypes were detected in the sample population. *BspM*I digestion of amplified products revealed a single fragment (161 bp) for TT genotype and 2 fragments (118 and 43 bp) for TC genotype (Figs 2 A and 2 B). No bulls of CC genotypes could be observed. Further confirmation of the respective genotypes was assessed through DNA sequencing results (Figs 1C, 2C). Table 1 represented the overall genotypes and allele frequencies. Allele A at G286A locus and allele C at T340C

Table 1. Genotype and allele frequency of G286A and T340C locus of GnRHR gene among selected bull population

Gene locus	Genotype frequency	Allele/gene frequency
G 286 A	GG: 0.94	G: 0.97
	GA: 0.06	A:0.03
	AA:0.00	
T340C	TT:0.83	T:0.92
	TC:0.17	C:0.80
	CC:0.00	

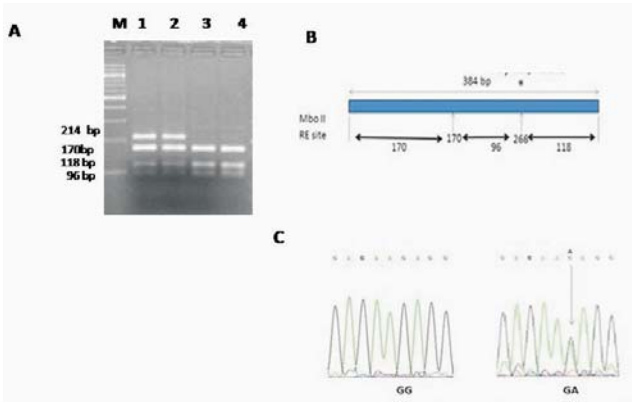

Fig. 1. A. PCR RFLP pattern of SNP G286A region of GnRHR gene. Lane 1and 2 : GA genotypes; Lane 3 and 4: GG genotypes; M: DNA ladder. B. *MboII* restriction enzyme digestion pattern. C. Chromatogram representation of GG and GA genotypes.

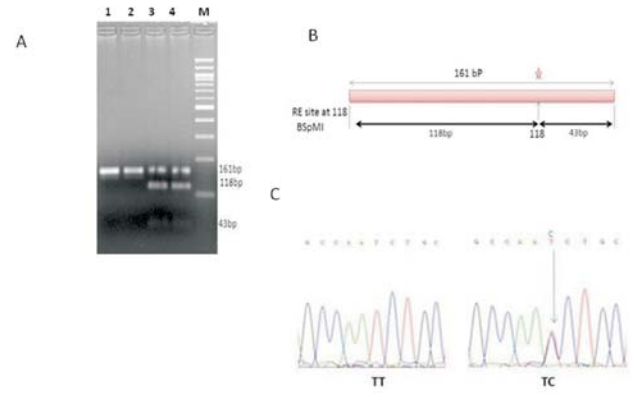

Fig. 2. A. PCR-RFLP pattern of SNP T340C region of GnRHR gene. Lane 1 and 2. TT genotypes; Lane 3 and 4. TC genotypes; M: DNA ladder. B. *BspMI* restriction enzyme digestion pattern. C. Chromatogram representation of TT and TC genotypes.

locus was observed to be rare alleles with the frequency of 0.03 and 0.08, respectively in the population under study. AA and CC genotypes could not be detected in the test population. It may perhaps due to the low frequency of allele A and allele Cat locus G286A and T340C, respectively. Consequently, a larger bull population needs to be screened to observe those rare genotypes. It is noteworthy to mention here that Yang *et al.* (2011) reported for low frequency of A (locus G286A) and C (locus T340C) alleles in 131 Chinese Holstein bulls than we observed in Frieswal. In this context it will be interesting to discuss that the Frieswal crossbred population has undergone seven generations of interbreeding and stabilized with a HF inheritance of around 62.5%. Therefore, from the study it may also be inferred that the frequency distribution of A and C allele will be in rare category in Sahiwal, the Indian zebu breed used to develop the crossbred Frieswal population. However, this statement should further be validated experimentally.

Table 2 represents the overall mean, standard deviation (S.D.) and ranges of each phenotypic traits related to semen quality parameters among the studied bull populations. The results of association analysis between GnRHR genotypes of polymorphic loci G286A and T340C with sperm quality traits are presented in table 3 and 4, respectively. In polymorphic locus G286A, although bulls with the GA genotype had higher SCON, MOT, PTM, viability, HOST than bulls with homozygous GG genotype, however, no significant difference ($P > 0.05$) was observed (Table 3). However, Yang *et al.* (2011) reported that Holstein bulls with the GA genotype had a significantly ($P < 0.01$) higher FMOT (Frozen Motility) than the GG genotype. However, in polymorphic locus T340C, Frieswal bulls with homozygote TT genotype showed significantly higher sperm concentration ($P < 0.05$) and lower semen volume ($P < 0.01$) compared to bulls with heterozygote TC genotype (Table 4). Yang *et al.* (2011) observed that heterozygote TC genotype had significantly higher sperm motility (MOT), semen volume per ejaculate (VOL), and lower abnormal spermatozoa rate (ASR) than homozygote TT genotype ($P < 0.05$). On the contrary, it was observed that TT genotype had a slightly higher IPM, PTM, viability and HOST response compare to TC genotype but there was no significant differences ($P > 0.05$) (Table 4).

Our preliminary observation thus may indicate that Frieswal bulls with TT genotypes had a favorable positive effect on sperm quality traits. This may be due to the reason

Table 2. Mean, standard deviation (S.D.) and ranges of each phenotypic trait related to semen quality parameters among Frieswal bulls

Traits	Sample size	Mean	S.D.	Minimum	Maximum
[a]Vol (ml)	96	4.57	1.43	1.57	9.91
[a] SCON (M/ml)	96	970.44	324.74	37.50	1879.78
[a]IPM (%)	96	42.99	15.93	0.00	67.92
[a]PTM (%)	87	30.75	12.38	10.00	54.00
Viability (%)	40	61.56	15.17	24.11	83.23
[a]HOST (%)	74	47.62	18.17	11.00	76.00

[a] Vol, Semen volume, SCON, sperm concentration; IPM, initial progressive motility; PTM, post-thaw motility; HOST, Hypo osmotic swelling test response.

Table 3. Effects of G286A SNP on semen quality traits of Frieswal bull

Genotypes	[a]Vol (ml)	[a]SCON (M/ml)	[a]IPM (%)	[a]PTM (%)	Viability (%)	[a]HOST (%)
GA (n=6)	3.74 ±0.50$_{ns}$	1040.45 ±97.69$_{ns}$	41.77 ±6.86$_{ns}$	29.33 ±7.05$_{ns}$	52.74 ±6.05$_{ns}$	37.42 ±5.76$_{ns}$
GG (n=90)	4.62 ±0.15$_{ns}$	965.78 ± 34.80$_{ns}$	43.07 ±1.68$_{ns}$	30.83 ±1.35$_{ns}$	62.02 ±2.49$_{ns}$	48.52 ±2.21$_{ns}$

Data shown here in mean ± standard error. Ns, nonsignificant. [a] Vol, Semen volume, SCON, sperm concentration; IPM, initial progressive motility; PTM, post-thaw motility; HOST, Hypo osmotic swelling test response

Table 4. Effects of T340C SNP on semen quality traits of Frieswal bulls

Genotypes	[a]Vol (ml)	[a]SCON (M/ml)	[a]IPM (%)	[a]PTM (%)	Viability (%)	[a]HOST (%)
TT (n=80)	4.24 ± 0.11$_{a}$	1003.47 ±36.61$_{c*}$	43.65 ±1.74$_{ns}$	31.01 ±1.44$_{ns}$	61.15 ±2.67$_{ns}$	46.50 ±2.39$_{ns}$
TC (n=16)	6.19 ± 0.14$_{b}$	870.44 ± 33.14$_{d*}$	39.70 ±4.43$_{ns}$	29.35 ±3.45$_{ns}$	63.19 ±5.72$_{ns}$	51.67 ±4.49$_{ns}$

Data shown here in mean ± standard error. Different lower case subscript letters ($_{a,b,c,d}$) of least squares mean within a row mean significant difference at $P<0.05$ and $*P<0.01$, ns: non-significant. [a] Vol, Semen volume, SCON, sperm concentration; IPM, initial progressive motility; PTM, post-thaw motility; HOST, Hypo osmotic swelling test response.

Table 5. Effects of combined genotypes (haplotypes) on semen quality traits of Frieswal bulls

Haplotypes	[a]VOL (ml)	[a]SCON (M/ml)	[a]IPM (%)	[a]PTM (%)	Viability (%)	[a]HOST (%)
GGTT (n=74)	4.28 ±0.11$_{a}$	1000.47 ±38.90$_{c}$	43.80 ±1.81$_{ns}$	31.14 ±1.47$_{ns}$	61.71 ±2.81$_{ns}$	47.55 ±2.55$_{ns}$
GATT (n=6)	3.74 ± 0.51$_{a}$	1040.45 ± 97.69$_{c}$	41.77 ±6.86$_{ns}$	29.33 ±7.05$_{ns}$	52.74 ±6.05$_{ns}$	37.42 ±5.76$_{ns}$
GGTC (n=16)	6.19 ± 0.49$_{b}$	805.30 ± 65.32$_{d}$	39.70 ±4.43$_{ns}$	29.35 ±3.45$_{ns}$	63.19 ±5.70$_{ns}$	51.67 ±4.49$_{ns}$

Data shown here in mean ± standard error. Different lower case subscript letters ($_{a,b,c,d}$) of least squares mean within a row mean significant difference at $P<0.05$, ns: non- significant. [a] VOL: Semen Volume, SCON : Sperm Concentration, IPM : Initial Progressive Motility, PTM: Post-thaw Motility and HOST: Hypo osmotic swelling test response.

that, as the mutations is synonymous, they perhaps increase the density of GnRHR on gonadotropes by affecting the expression of the GnRHR gene and the stability of the GnRHR transcription which may leads to increase of the gonadotrophin concentrations (Leanos-Miranda *et al.* 2002, Schubert *et al.* 2000).

It is well studied that the genotype effect of single SNP may be predisposed by other SNPs and thus the genotype blending effect is an indication of interactions of multiple SNPs (Zheng *et al.* 2011). Thus, the investigation of genotype combination is better for the analysis of the single SNP. In the present study, three kinds of combined genotypes of two SNPs combination (G286A and T340C) were observed among the bull herds. There was significant ($P<0.05$) association between the combined genotypes of two SNPs and certain semen quality traits. Statistic results exhibited that the bulls with the genotype GGTC had the significantly ($P<0.05$) highest semen volume and lowest concentration (Table 5).

The results of our preliminary observations may thus indicate that SNPs at GnRHR may be favorable biomarkers for selecting bulls with better semen quality traits. These findings may need to be further validated with large number of samples. Additional research also needs to be established for investigating the expression level of the GnRHR and their association with the semen quality performances.

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REFERENCES

Albertson A J, Navratil A, Mignot M, Dufourny L, Cherrington B and Skinner D C. 2008. Immunoreactive GnRH type I receptors in the mouse and sheep brain. *Journal of Chemical Neuroanatomy* **35**: 326–33.

Deb R, Kumar S, Singh U, Tyagi S, Mandal D K, Sengar G, Singh R, Kumar M and Sharma A. 2013. Evaluation of three bovine Y specific microsatellite loci as an alternative biomarkers for semen quality traits in crossbred bull. *Animal Reproduction Science* **142**: 121–25.

Ferreira G, Silveira L, Barbosa T E, Latronico AC. 2010. Genetics basis for GnRH-dependent pubertal disorders in humans. *Molecular Cell Endocrinology* **324**:30–38.

Ganguly I, Gaur G K, Kumar S, Mandal D K, Kumar M, Singh U, Kumar S and Sharma A . 2013. Differential expression of protamine 1 & 2 genes in mature spermatozoa of normal and motility impaired semen producing Frieswal (HF × Sahiwal) bulls. *Research in Veterinary Science* **94**: 256–62.

Kumar S, Deb R, Singh U, Ganguly I, Mandal D K, Tyagi S, Kumar M, Sengar G, Sharma S, Singh R, Singh R. 2015. Bovine Circadian Locomotor Output Cycles Kaput (CLOCK) and Clusterin (CLU) mRNA quantitation in ejaculated crossbred bull spermatozoa. *Reproduction in Domestic Animals* DOI: 10.1111/rda.12522.

Leanos-Miranda A, Janovick J A and Conn P M. 2002. Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone receptor-mediated hypogonadotropichypogonadism. *Journal of Clinical Endocrinology and Metabolism* **87**: 4825–28.

Lin C L, Ponsuksili S, Tholen E, Jennen D G, Schellander K, Wimmers K. 2006. Candidate gene markers for spermquality and fertility of boar. *Animal Reproduction Science* **92**: 349–63.

Milazzotto M P, Rahal P, Nichi M., MirandaNeto T, Teixeira L A, Ferraz J B S, Eler J P, Campagnari F, Garcia J F. 2008. New molecular variants of hypothalamus–pituitary–gonad axis genes and their association with early puberty phenotype in *Bos taurus indicus* (Nellore). *Livestock Science* **114**: 274–79.

Schubert U, Anton LC, Gibbs J, Norbury C C, Yewdell J W and Bennink J R .2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**: 770–74.

Yang W C, Li S J, Xie Y H, Tang K Q, Hua G H, Zhang C Y, Yang L G. 2011. Two novel SNPs of the type I gonadotropin releasing hormone receptor gene and their associations with superovulation traits in Chinese Holstein cows. *Livestock Science* **136**: 164–68.

Zheng X, Ju Z, Wang J, Li Q, Huang J, Zhang A, Zhong J, Wang C. 2011. Single nucleotide polymorphisms, haplotypes and combined genotypes of LAP3 gene in bovine and their association with milk production traits. *Molecular Biology Reports* **38**: 4053–61.