



Decoding HF crossbred bull fertility: Candidate gene markers contributing significantly to the phenotypes

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ABSTRACT

Bull fertility is an important trait determining conception in cows and subsequently, influencing other economic traits. Sire conception rate has been used as a reliable predictor of bull fertility and is often considered as a pseudo-phenotype for selection. However, in order to make substantial genetic improvements in the trait, information related to genetic markers for major candidate genes associated with bull fertility is indispensable. Based on a candidate gene approach, this study aimed to identify SNP markers in AKR1B1 and INCENP genes having a major role in determining bull fertility using Sire Conception Rate (SCR) as a pseudo-phenotype. A total of 3308 artificial insemination records of 40 HF crossbred bulls belonging to the different sets of progeny testing programme were utilized for estimating SCR. Seminal parameter traits were also recorded for all the bulls. The polymorphisms detected in AKR1B1 and INCENP genes was then associated with SCR and seminal traits. The estimated SCR ranged from -16.0 to +17.4. The least-squares mean estimates for post thaw motility (PTM), acrosomal integrity, percent live spermatozoa and plasma membrane integrity of frozen thawed semen samples were estimated as 49.68 ± 0.50 , 62.43 ± 0.50 and 57.91 ± 0.42 , $51.11 \pm 1.10\%$ respectively. As far as PCR-RFLP and sequencing studies were concerned, AKR1B1 gene exhibited a polymorphism in the intron 7 with A>G change at position 333 while promoter region of INCENP gene showed change T>G at position 79. Association analysis revealed a significant association of the genotypes of AKR1B1 gene with SCR and those of INCENP gene with PTM. Breeding HF crossbred bulls with AA genotype for AKR1B1 gene and GG genotype for INCENP gene were desirable in the herd for higher bull fertility. SNP markers associated with bull fertility identified in this study may be included in marker-based selection for the trait after proper validation in future.

Keywords: AKR1B1, Bull fertility, Genetic markers, HF crossbred bull, INCENP, Seminal parameters, Sire conception rate

The realization that dairy animal fertility plays an important role alongside milk production in the farm economics dawned on breeders and farm managers in the late 1990s, when failure to conceive topped the list of causes for involuntary culling of cattle (Bascom and Young 1998). However lately, there has been a growing awareness regarding bull fertility as an indispensable factor determining conception in cows (Rezende *et al.* 2018). Numerous research findings have consolidated the view that differences in bull fertility manifested in its semen quality and Sire Conception Rate (SCR), could have enormous implications on the sperm fertilizing ability (Ortega *et al.* 2018), viability of preimplantation embryo (Kropp 2014) and hence, the overall conception rate in dairy cattle (DeJarnette *et al.* 2014, Jamrozik *et al.* 2005,

Nagamine and Sasaki 2008).

Traditionally, bull fertility in animal breeding was determined based on Estimated Relative Conception Rate (ERCR), which used non return rate at 70 days for first service as a measure of the success or failure of first service and subsequently, the conception rate (AIPL Research Report 2008). Introduction of SCR in 2008 improved bull fertility estimation procedure as it included multiple services instead of first service only to estimate conception rate (Kuhn and Hutchison 2008). Since then, SCR is used as a reliable phenotypic predictor of bull fertility and is often considered as a pseudo-phenotype (a projection of phenotype closest to the genotype) for determining associations between SCR and various factors, parameters or genetic markers (Norman *et al.* 2011, Han and Peñagaricano 2016, Abdollahi-Arpanahi *et al.* 2017, Ortega *et al.* 2018). Studies on genome, proteome and metabolome to identify genetic markers and biomarkers for sperm fertility and seminal plasma further consolidate this point (Viana *et al.* 2018, Taylor *et al.* 2018b, Maheshwarappa *et al.* 2019, Menezes *et al.* 2019).

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The crux of the matter lies in the fact that identification of genetic markers related to SCR and seminal quality parameters would be an important finding in accounting for genetic component of bull fertility (Mishra *et al.* 2013, Rezende *et al.* 2018). Till date, various candidate genes related to bull fertility traits have been discovered, AKR1B1 (Aldo-Keto Reductase Family1 Member B1) and INCENP (Inner Centromere Protein) genes, being two of them. AKR1B1 is an enzyme which catalyses the conversion of glucose to sorbitol in carbohydrate metabolism and subsequently, sorbitol is metabolised by sorbitol dehydrogenase to produce fructose. Fructose acts as the ultimate source of energy to the sperm cells, thus affecting sperm motility and maturation (Chung and LaMendola 1989, Kia 2007). On the other hand, INCENP gene has its role in cell division, sister chromatid separation and cytokinesis (Resnick *et al.* 2009). So, chromatid separation in spermatogenesis to produce normal sperms is regulated by this gene (Zhuang *et al.* 2014). The genetic polymorphism in INCENP gene has been implicated as a factor regulating sperm quality by various studies (Hering *et al.* 2014, Liu *et al.* 2016).

In India, till date, bulls are evaluated based on milk production of their daughters through progeny testing (National Dairy Plan Phase-I 2018) and not on the basis of their own fertility criteria like SCR or molecular marker information. This study is the first in India to integrate the molecular marker information of important candidate genes AKR1B1 and INCENP with phenotypic bull fertility determinants like seminal parameters and pseudo-phenotype SCR in HF crossbred bulls (Holstein Friesian × Tharparkar).

MATERIALS AND METHODS

Source of information: This study was conducted on 40 HF crossbred bulls (5/8HF × 3/8Tharparkar) belonging to 12 different sets of Progeny Testing programme, being carried out at ICAR-NDRI, Karnal. Data pertaining to the 3308 AI records of the HF crossbred bulls was collected from history cum pedigree sheets maintained at Livestock Research Centre of the institute. The climate is subtropical

in nature with the minimum temperature falling near the freezing point in winter months, whereas the maximum temperature goes up to around 45°C in summer. The annual rainfall is about 760 to 960 mm, most of which is received during the months of July and August. The relative humidity ranges from 41 to 85%. Based on this, it can be deduced that the bulls were exposed to extreme climatic conditions due to wide variations in temperature and humidity.

Isolation of genomic DNA and PCR amplification: Genomic DNA was isolated from blood samples of HF crossbred bulls by phenol:chloroform extraction method (Sambrook and Russel 2001). After ascertaining the quality and quantity of DNA, PCR amplification was carried out using reported primers for intron 7 of AKR1B1 gene and promoter region of INCENP genes (Table 1).

The reaction mixture for PCR comprised 3 µl of genomic DNA, 12.5 µl of 2× master mix (Dream Taq, Thermo Fisher Scientific), 0.5 µl forward primer, 0.5 µl reverse primer, 0.3 µl MgCl₂ and 8.2 µl of nuclease free water (NFW) for AKR1B1 (8.5 µl of NFW for INCENP genes). The cycling condition for the two genes included an annealing temperature of 56.4°C for AKR1B1 and 57°C for INCENP genes, respectively.

PCR-RFLP and DNA sequencing: PCR product was digested with NdeI and AluI restriction enzymes (RE) for AKR1B1 and INCENP genes, respectively at 37°C in a water bath for 12-14 h in order to carry out Restriction Fragment Length Polymorphism (RFLP). After RE digestion, the products were resolved by gel electrophoresis on 3% agarose gel at 70 volts for 1 h. The identified SNPs through RFLP were reconfirmed by Sanger DNA sequencing and further, by multiple sequence alignment using Bioedit software. The sequence was aligned with *Bos taurus* reference sequence (NC_037331.1).

Estimation of conception rate (CR) and sire conception rate (SCR): HF crossbred bulls with minimum of 40 AI records were considered for estimation of CR. After normalizing the data, the descriptive statistics for estimating mean and standard error of the different parameters was done by using standard statistical procedure (Snedecor and Cochran 1994). To assess the effect of non-genetic factors

Table 1. Primer sequence and PCR reaction conditions for AKR1B1 and INCENP genes

Gene	Primer	Annealing Temp.	Reference
AKR1B1 (796 bp)	F: 5' ACCAGGGCTTACCTGGAAGT 3' R: 5'GGTCAATGGGCCTTAGGATT 3'	56.4°C	Kia (2007)
INCENP (205 bp)	F: 5'GCCTACAGCCTGAAGAAG 3' R: 5'AGTGCTGTCCACAGACCA 3'	57°C	Liu <i>et al.</i> (2016)
PCR steps			
	AKR1B1	INCENP	
Initial denaturation	95°C for 4 min	94°C for 3 min	
Denaturation	94°C for 40 sec	94°C for 30 sec	
Annealing	56.4°C for 45 sec	57°C for 30 sec	
Extension	72°C for 1 min	72°C for 1 min	
Final extension	72°C for 10 min	72°C for 10 min	
No. of cycles	35	35	

affecting conception rate, the Least-squares fixed model (Harvey 1990) was used.

$$Y_{ijkl} = \mu + S_i + b_1 (BW_j - BW) + b_2 (AF_k - AF) + e_{ijkl}$$

where Y_{ijkl} , conception rate of l^{th} bull with i^{th} season of first freezing, j^{th} birth weight and k^{th} age at first freezing of semen; μ , overall mean; S_i , effect of i^{th} season of first freezing; b_1 , Regression of birth weight of bull on CR; BW_j , Birth weight of j^{th} bull; BW , Average birth weight of bulls; b_2 , Regression of age of bull at first freezing of semen on CR; AF_k , Age of k^{th} bull at first freezing of semen; AF , Average age of bulls at first freezing of semen; e_{ijkl} , random residual $\sim$ NID (0, σ^2_e). Seasons were classified into four subclasses depending on the climatic variation in Karnal throughout the year: Winter (December to March), Summer (April to June), Rainy (July to September) and Autumn (October to November) (Singh 1983). The difference in the mean of the season subclasses were compared by Duncan's multiple range test (Kramer 1957). SCR was calculated as the deviation of individual CR of bulls from the average CR of the herd.

Semen analysis: Seminal parameters were analysed from frozen thawed semen straws and sampling was done in all four seasons as mentioned earlier. A minimum of 12 semen straws from each bull in three replications per season were used for semen analysis. Straws were thawed at 37°C for 50 sec and post thaw motility (PTM) was estimated under the phase contrast microscope. The per cent live spermatozoa was determined by adopting differential staining technique using Nigrosin-Eosin stain (NE) (Campbell *et al.* 1956). Acrosome integrity as a measure of percent normal acrosome, was estimated by Giemsa stain (Watson 1975). Hypo-osmotic Swelling Test (HOST) was performed (Jeyendran *et al.* 1984) which gives an idea of the spermatozoal fertilizing capacity *in vitro*.

Correlation between seminal parameters: Phenotypic correlation was calculated between four seminal quality parameters viz. post thaw motility, percent live spermatozoa, acrosomal integrity and HOST. Since the conception rate of bulls was estimated based on semen straws used in

different seasons and years, it was not correlated with any of the seminal parameter.

Association analysis: Association analysis between genotypes of genes (AKR1B1 and INCENP) and SCR as well as seminal parameters as a measure of bull fertility was done using IBM SPSS software version 22. The following generalized linear model was used, considering genotype as a fixed factor:

$$Y_{ij} = \mu + G_j + e$$

Where Y_{ij} , SCR or seminal parameters of j^{th} bull with i^{th} genotype; μ , overall mean; G_j , fixed effect of j^{th} genotype; e , random residual error $\sim$ NID (0, σ^2_e).

RESULTS AND DISCUSSION

Descriptive statistics of recorded phenotypic traits: The average CR in the herd was estimated to be 43.09 $\pm$ 1.39% while it ranged from 24.39 to 59.09%. Before adjusting the effect of covariables like birth weight and age at first freezing, their mean values were determined. The average birth weight of the bulls was 30.86 $\pm$ 0.77 kg with the lowest and highest recorded birth weights as 22 kg and 42 kg, respectively. Average age at first freezing (AFF) ranged from 1.14 years to 4.52 years with an average of 2.80 $\pm$ 0.11 years.

Adjustment of non-genetic factors affecting CR and estimation of SCR: The overall least-squares mean for CR in HF crossbred bulls after adjusting the data, was 43.62 $\pm$ 1.36%. Season of first freezing of semen was found to be having a significant ($p < 0.05$) effect on CR in HF crossbred bulls while the other factors did not exhibit any significant impact (Table 2). CR (%) was higher in case of bulls whose first semen was frozen in autumn (48.63) season followed by summer (45.04), rainy (40.68) and winter (40.15) suggesting that semen should be preferably frozen in autumn for improving the CR. Post adjustment of effect of season of first freezing, SCR was calculated from CR estimates and it ranged from -16.0 to +17.4 with the most superior bull having 17.4% higher CR than the average of the herd.

Evaluation of seminal quality parameters and their correlation: The average post thaw motility (PTM), acrosomal integrity, percent live spermatozoa and plasma membrane integrity (HOST) of frozen semen samples (in %) were 50.68 $\pm$ 0.56 (range 32.00 to 58.75), 62.43 $\pm$ 0.59 (range 37.00 to 72.75), 58.20 $\pm$ 0.69 (range 45.50 to 69.00) and 51.11 $\pm$ 1.10 (range 32.00 to 60.65), respectively. The least squares mean obtained in different seasons revealed that comparatively better estimates were obtained in winter and autumn seasons (October to March) for PTM (51.85 $\pm$ 1.06 and 52.58 $\pm$ 1.10%), acrosomal integrity (65.85 $\pm$ 1.03 and 65.58 $\pm$ 1.09%), non-eosinophilic count (62.50 $\pm$ 1.05 and 58.38 $\pm$ 1.08%) and HOST (56.85 $\pm$ 1.06 and 51.28 $\pm$ 1.02%), respectively (Table 3) as compared to summer and rainy seasons which prove detrimental for sperm quality and motility. Season of first freezing had a highly significant effect ($p < 0.01$) on all the semen quality parameters.

Table 2. Least squares means and SE of conception rate in HF crossbred bulls

Effect	Conception rate (CR)
Overall mean (μ)	43.62 $\pm$ 1.36 (40)
<i>Season of first freezing</i>	
Winter	40.15 $\pm$ 1.85 ^a (17)
Summer	45.04 $\pm$ 2.50 ^b (10)
Rainy	40.68 $\pm$ 3.02 ^a (7)
Autumn	48.63 $\pm$ 3.41 ^c (6)
Age at first freezing (b_1)	0.27 $\pm$ 1.81 (40)
Birth weight (b_2)	-0.12 $\pm$ 0.28 (40)

Figures in parenthesis are the no of observations. a, b, c similar superscript indicates non-significant difference between seasons. a,b,c Dissimilar superscript indicates significant difference between seasons. b_1 is regression of age at first freezing on the trait, b_2 is regression of birth weight on the trait.

High phenotypic correlation was found between different seminal parameters, with PTM and acrosomal integrity showing the maximum correlation (0.80) followed by correlation between PTM and percent live spermatozoa (0.69) then between acrosomal integrity and percent live spermatozoa (0.68) respectively (Table 4).

Genetic polymorphism in the targeted regions: The standardized conditions and optimization of PCR program gave an amplified product of 796 bp fragment of the Intron 7 region of AKR1B1 gene and 205 bp fragment of the promoter region of INCENP gene. PCR-RFLP of the same regions with NdeI (recognition sequence – CATATG) and AluI (recognition sequence – AGCT) revealed two genotypes – AG (796, 463 and 333 bp) and AA (463 and 333 bp) in case of the former (Fig. 1) and three genotypes–TT (205 bp), GT (205, 126 and 79 bp) and GG (126 and 79 bp) in case of latter (Fig. 2). For reconfirmation of RFLP results, samples were sent for Sanger sequencing and chromatogram analysis followed by ClustalW multiple sequence alignment, revealed adenine to guanine change at position 333 (A333G) in intron 7 region of AKR1B1. Similarly, for promoter region of INCENP gene, chromatogram analysis showed Thymine to Guanine change at position 79 (T79G), thus verifying RFLP findings.

Association of bull fertility determinants with the identified polymorphisms: For AKR1B1 gene, AA genotype was predominant in the herd with genotypic frequency of 0.85 while for AG genotype, the genotypic frequency was 0.15. Also, the allele frequency was quite higher for allele A (0.93) than G (0.07), suggesting that it was predominant in the HF crossbred bulls taken for the study. Importantly, SCR was found significantly associated ($p < 0.05$) with the AKR1B1 genotypes and genotype AA was desirable in the herd with higher SCR (3.68% more than the average) as compared to AG genotype (3.98% less than the herd average). Association analysis of genotypes and seminal parameters revealed that the difference between AA and AG genotype bulls was significant only with respect to

percent live spermatozoa which was significantly ($p < 0.05$) higher in AA genotype (58.50%) bulls than AG genotype (56.57%) ones. Statistically, no significant difference was found in rest of seminal parameters evaluated in the study whereas in general, higher fertility was observed in AA genotype bulls as compared to AG genotype bulls for AKR1B1 studied region.

For INCENP gene, allele T (0.63) was predominant than allele G (0.37). Also, the genotypes of SNP T79G were found to be significantly ($p < 0.05$) associated with the seminal post thaw motility and further, GG genotype was having maximum post-thaw motility (52.6%). For acrosomal integrity, percent live spermatozoa and HOST, no significant difference was seen among the genotypes (Table 5).

This study is a part of plethora of studies carried out on various aspects of bull fertility till date. There was considerable variation in the CR which could be attributed to the difference in the age of bulls at semen collection, different seasons of semen collection and various management factors in the farm. Bull fertility is a multifactorial trait and is regulated by various environmental factors along with the genetics (Petherick 2005). So, studies have intended to determine the effect of any non-genetic factor playing an important role in bull fertility dynamics and hence, nullify the same. On similar lines, a study (Rekwot *et al.* 1987) reported that after adjusting the data for non-genetic factors, better ejaculate quality was obtained during rainy season in Zebu, Friesian and their crossbred bulls. Consequently, higher conception rates were reported for semen collected and frozen during the rainy season in their A.I. programme. In contrast, a study on Norwegian Red breed of cattle (Haugan *et al.* 2005), reported that season of semen collection and freezing had no effect on 56d non return rate. In one of the studies conducted in similar climatic conditions as the present study (Fiaz *et al.* 2009), it was concluded that stressful summer season deteriorated the quality of semen obtained from both Holstein Friesian and Jersey breeding bulls maintained under sub-tropical

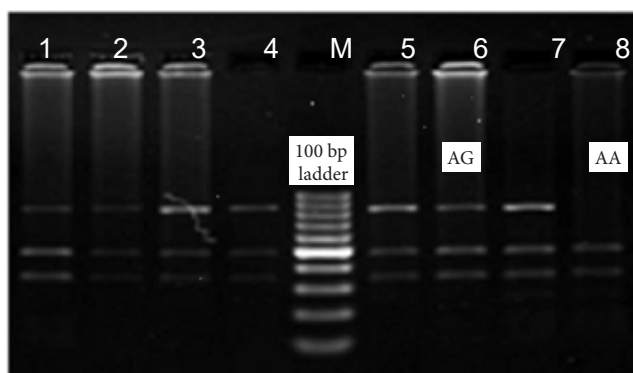


Fig. 1. RFLP band pattern of AKR1B1 gene (Intron 7). Lane 1,2,3,4,5,6,7: AG Genotype (796 bp, 463 bp and 333 bp); Lane 8: AA Genotype (463 bp and 333 bp).

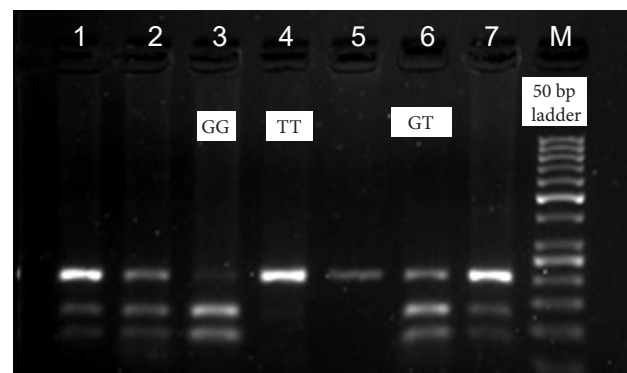


Fig. 2. PCR-RFLP of INCENP gene (Promoter region) in K. F. bulls. Lane 1,2,6,7: GT Genotype (205 bp, 126 bp and 79 bp); Lane 3: GG Genotype (126 bp and 79 bp); Lane 4: TT Genotype (205 bp); Lane 5: PCR Product (205 bp); Lane M: 50 bp ladder.

Table 3. Least squares means values for seminal parameters in HF crossbred bulls

Effect	Post thaw motility (PTM)	Acrosomal Integrity (AI)	Non-Eosinophilic sperm Count	HOST
Overall mean (μ)	49.68 $\pm$ 0.50 (160)	62.43 $\pm$ 0.50 (160)	57.9 $\pm$ 0.42 (160)	51.11 $\pm$ 1.10 (160)
Season of freezing				
Winter	51.85 ^c $\pm$ 1.06 (40)	65.85 ^c $\pm$ 1.03 (40)	62.50 ^c $\pm$ 1.05 (40)	56.85 ^c $\pm$ 1.06 (40)
Summer	44.88 ^a $\pm$ 1.01 (40)	56.88 ^a $\pm$ 1.02 (40)	52.69 ^a $\pm$ 1.03 (40)	46.68 ^a $\pm$ 1.03 (40)
Rainy	49.41 ^b $\pm$ 1.07 (40)	61.41 ^b $\pm$ 1.07 (40)	59.50 ^b $\pm$ 1.06 (40)	50.82 ^b $\pm$ 1.09 (40)
Autumn	52.58 ^c $\pm$ 1.10 (40)	65.58 ^c $\pm$ 1.09 (40)	58.38 ^b $\pm$ 1.08 (40)	51.28 ^b $\pm$ 1.02 (40)

Figures in parenthesis are the no of observations. a, b, c similar superscript indicates non-significant difference between seasons. a,b,c Dissimilar superscript indicates significant difference between seasons.

Table 4. Phenotypic correlation between different seminal parameters

	Percent live spermatozoa	PTM	Acrosomal integrity	HOST
SCR	1			
PTM	0.69	1		
AI	0.68	0.80	1	
HOST	0.42	0.54	0.60	1

environment of Pakistan. We also found a higher percentage of successful CR, when semen was frozen in winter and short autumn season. Good weather conditions just after the rainy season and in winters facilitate the availability of good quality fodder combined with better managerial conditions in the farm, ultimately enhancing the semen quality. We would like to complement this with the further information that the frozen semen straws were used in the herd for AI, 2 to 3 months after collection, which implied that semen frozen in autumn season was used in winters, when the overall performance of HF crossbred animals is at its peak in terms of production as well as reproduction. On the other hand, semen collected in winter season is usually of very high quality. So, the deteriorating impact of summer season is somewhat mitigated. The little increase in the CR of bulls after adjusting data for season of first freezing in our study could be attributed to the fact that bulls were not able to perform to their full potential in particular seasons. The variation in CR seen in different seasons could also be due to the difference in the number of observations in each subclass. Overall, it could be deduced that season is an important source of variation in achieving good conception rate in HF Crossbred bulls in our farm. The birth weight of bulls can never be ignored

as it is indispensable for attaining early sexual maturity and selecting bulls with good birth weight can be starting point in genetic improvement of bulls (Mir *et al.* 2015). Regarding birth weight of bulls, several studies conducted in our farm have suggested the importance of birth weight of bulls in relation to conception rate (Naha *et al.* 2015).

SCR, a reliable predictor of bull fertility, was based on available data and estimates were adjusted for various non-genetic factors as explained in evaluation models (Kuhn *et al.* 2008). Literature regarding the estimation of SCR in HF crossbred bulls is scanty, though few workers have reported SCR ranging from -10.66 % to +6.80% (Peñagaricano *et al.* 2012) and -4% to +7% (Kuhn *et al.* 2006) in Holstein and Jersey bulls, respectively. The comparatively larger variability in SCR in our study could be due to variation in the seminal attributes and AI records of different HF crossbred bulls used. Other probable reasons could be higher genetic variability in our herd and comparatively small sample size in our study.

Seminal parameters have been implicated as an important factor affecting bull fertility. Available reports suggest that >50% HF crossbred young bulls introduced for semen collection had problems with semen parameters, libido and cryotolerance of spermatozoa (Mandal and Tyagi 2004, Mukhopadhyay *et al.* 2010). None of the seminal attribute can be used for predicting complete fertility status of an ejaculate, however estimation of post thaw motility is increasingly being used as yardstick to assess the success of frozen semen technology and overall conception rate of bull semen. The PTM estimated in our study was more than >50% which is above the minimum standards for production of bovine semen (National Dairy Plan Phase-I 2018).

Table 5. Association of AKR1B1 and INCENP genotypes with fertility traits in HF crossbred bulls

Genotype	No of bulls	SCR	PTM	Acrosomal integrity	Percent live spermatozoa	HOST
<i>INCENP Gene (T79G)</i>						
TT	16	-0.49	47.9 ^a	63.23	59.00	52.02
TG	18	-0.65	50.2 ^{ab}	62.24	59.05	51.27
GG	6	0.05	52.6 ^b	61.95	57.84	53.71
<i>AKR1B1 Gene (A333G)</i>						
AG	6	-3.98	49.74	62.53	56.57	51.73
AA	34	3.68	50.24	62.19	58.50	52.27

In another detailed study on seminal parameters of HF and Jersey crossbred bulls (Mathur *et al.* 2014), estimated post thaw motility in crossbreds (HF, Jersey, and other crosses) was 50.85% and the overall mean post-thaw motility was 51.02%. Another study (Zodinsanga *et al.* 2015) reported around 40% PTM in 14 cross-bred and 12 pure bred bulls. The reasons for variation in post thaw motility might be due to different season of collection of semen, age of bulls and cryopreservation methods.

Acrosomal integrity plays a very important role during capacitation reaction and is highly important in regard to outcome of successful fertilization. Although maximum limit for acrosome alterations is not fixed, it is reported that the samples should not contain more than 40% of spermatozoa with acrosomal alterations (Selvaraju *et al.* 2016). Almost similar estimates of average percentage of acrosome integrity have been reported by Thomas *et al.* (1997), Farooq *et al.* (2013). As far as eosinophilic sperm count is concerned, our study is keeping in the trend with earlier studies (Goswami *et al.* 1991, Fiaz *et al.* 2009, Bhakat *et al.* 2014) suggesting that total percent live spermatozoa was significantly ($p < 0.05$) lowered during hot humid summer season. A viable sperm with functional integrity of plasma membrane is important for retaining its fertilizing ability (Rasul *et al.* 2001). Several workers (Thundathil *et al.* 2002, Srivastava and Kumar 2006) have reported higher estimates of HOST positive spermatozoa than the present study. There is also one report of HOST value being $73.76 \pm 0.47\%$ in Frieswal (HF $\times$ Sahiwal) bulls (Chauhan *et al.* 2017). Seminal parameters are expected to be correlated strongly with each other owing to the fact that sperm plasma membrane is a continuous structure comprising of head, mid-piece and tail (Karp 2009). Also, membrane integrity along with structural stability of the inner content is critical for the viability of spermatozoa (Rodriguez-Martinez 2007). So, functionally normal sperm with intact membranes is most likely to have optimum motility (Brito *et al.* 2003). Moreover, higher the percentage of live spermatozoa, higher is the expected post-thaw motility. Our findings were in accordance with various studies on the interrelationship between different seminal parameters, where medium to high correlation was found between the traits (Kumar 2004, Kirk *et al.* 2005, Lodhi *et al.* 2008). Unfortunately, these seminal parameters exhibit a small part of the differences seen in fertility among dairy sires (Parkinson 2004). The field fertility rate has been evaluated by many workers (Oliveira *et al.* 2012, Allouche *et al.* 2017) by considering different fertility traits and it has been found to vary amongst the bulls to the extent of 10-20% suggesting that these evaluations were not sufficient to predict fertility (Binsila *et al.* 2017). Integrating this information with newer fertility traits and genomic data will improve the accuracy of predicting bull fertility.

So, our attempt was to incorporate genetic marker information along with the phenotypic parameters of bull fertility. In a similar study in Chinese Holstein bulls (Liu

et al. 2016), SNP T79G (studied in the present study as well) of INCENP gene was found to be having correlation with initial sperm motility. For AKR1B1 gene, the same SNP(A>G) of AKR1B1 gene (at same position as in this study) was studied in 11 different cattle breeds (Limousin, Gelbvieh, Blond d'Aquitaine, Salers, Vorderwälder, Hinterwälder, Charolais, RedAngus, Piemontese, Pinzgauer and Galloway). (Kia 2007) reported in their study was AA (0.66) and AG (0.34). Frequencies of the allele 'A' and 'G' in their population were 0.82 and 0.18, respectively, which are in accordance with the allele frequency estimates obtained in our studied population. The same SNP was having a significant association with sperm survivability and motility traits, in line with our findings. Moreover, for sperm survivability and motility, animals with genotype AA had higher performance compared to genotype AG (49.4 vs. 48.2 and 71.4 vs. 59.7%, respectively). Genotype AA was predominant in our herd as well suggesting that selection for improved bull fertility is going on in the herd.

This substantiates our finding that the same marker of AKR1B1 gene could be incorporated in our selection criteria for improving the breeding efficiency of HF crossbred bulls in our farm. We are continuing this study to further follow-up the sons of the sires to validate our association findings. This study is novel from the perspective that the two SNPs (A333G and T79G) in AKR1B1 and INCENP genes were identified and their association with several bull fertility traits was determined for the first time in HF crossbred bulls of the country.

Now a days, bull fertility is gathering attention as an indispensable trait with SCR and seminal parameters serving as reliable phenotypes for identifying genetic markers associated with it. This study opens up the prospects of using SCR, in addition to seminal parameters for determining fertility in HF crossbred bulls in India. In future, bulls may be selected by giving a weightage to the genetic variation in the form of identified genetic markers related to these bull fertility traits along with the phenotypic traits. The SNPs identified in AKR1B1 and INCENP genes (A333G and T79G respectively) in the present study could be employed in the selection programmes after validation of their performance in the sons and grandsons of bulls.

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