RESEARCH PAPER | Veterinary Science | Volume : 5 | Issue : 8 | August 2015 | ISSN - 2249-555X

Molecular association of Y-chromosome specific genes with semen production traits in crossbred Jersey and crossbred Holstein Friesian bulls

KEYWORDS: SRY, MAS, SNP and CBJY bulls

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ABSTRACT *The present study was carried out to assess the polymorphism on Y-specific genes such as Sex determining region on Y- chromosome (SRY), Testis specific protein, Y- encoded(TSPY), Ubiquitin specific peptidase 9-Y linked (USP9Y) and DEAD box polypeptide 3-Y chromosome (DDX3Y) and their association with semen production traits in crossbred Jersey and crossbred Holstein Friesian bulls. Blood samples (5ml) collected from CBJY (80 samples) and CBHF (12 samples) bulls were used for this study. All the four Y-chromosome specific genes were highly conserved and they did not exhibit any polymorphism except SRY gene. The single nucleotide polymorphism at 641th position (G>T transversion) of SRY gene was associated with semen volume and sperm concentration in crossbred Jersey bulls. The results suggested that molecular markers associated with sperm quality traits could be used in marker assisted selection of breeding bulls.*

INTRODUCTION

Modern breeding programmes use artificial insemination with less number of breedable males for improving the economically important traits. However, some of the priced males have a low fertility even though the classical semen parameters (*i.e.* semen volume, sperm concentration and motility) are normal. Therefore, it is important to develop molecular tools to accurately estimate fertility levels among breeding bulls. In general, bull reproduction is an intricate physiological process comprised of sex differentiation, sexual maturation and determination. Each of these steps is guarded by a number of proteins with certain genetic basis. Hence, the genes encoding these processes can be considered as potential candidate gene markers for bull fertility.

The Y-chromosome represents only 2 to 3 per cent of the haploid genome. The X- and Y-chromosomes originated from a pair of autosomes around 300 million years ago among reptiles, much before mammals arose. The lack of recombination between X- and Y-chromosomes is thought to be responsible for the decay of Y-chromosome linked genes and this hypothesis seems to explain the small size of Y-chromosome as compared to X-chromosome[1]. During evolution, the Y-chromosome acquired a large number of testes-specific genes responsible for spermatogenesis and male reproduction related functions. Hence, deletion of any of these genes or structural change of gene sequence could result in altered fertility in bulls. Molecular techniques are used for detecting the variation or polymorphisms existing among bulls in the specific region of the DNA. Only limited studies have been carried out on the association of Y-specific gene polymorphism with semen production traits and the results were inconsistent. In India to date, there is dearth of studies on the expression variation of Y-specific genes in Crossbred Jersey (CBJY) and Crossbred Holstein Friesian (CBHF) bulls and their association with semen quality traits. Therefore, the present study was aimed to investigate the allelic frequencies of four Y-specific genes and its relationship with semen production traits of CBJY and CBHF bulls.

MATERIALS AND METHODS

Blood samples (5ml) were collected from CBJY (80 samples) and CBHF (12 samples) bulls of three frozen semen stations in Tamil Nadu, India and stored at 4°C till further processing. Genomic DNA was extracted using standard Phenol-Chloroform extraction procedure (Sambrook et. al., 1989).

Four Y-specific genes (with an accession number, Gene ID, number of base pairs and number of exons) such as (i) Sex determining region on Y- chromosome (NM_001014385, 280931, 690 bp and 1); (ii) Testis specific protein, Y- encoded (NM_001244608, 281554, 3206 bp and 7); (iii) Ubiquitin specific peptidase 9-Y linked (NM_0011455091, 100271721, 13485 bp and 7) and (iv) DEAD box polypeptide 3-Y chromosome (NM_0011725951, 783057, 10219 bp and 17) were chosen for the present investigation.

PCR primers were designed using an online software tool ("Primer3" input version 0.4.0) for these Y- specific genes based on the *Bos taurus* gene sequence available in the GenBank to amplify the expressed regions. The primers were analysed in "Oligo Analyzer" 1.0.3 software to find the GC content, self-annealing and primer loops. The annealing temperatures of each primer set (forward and reverse) were standardized by carrying out gradient PCR (Table 1).

The PCR mixture consisted of 10x PCR buffer, 1.5mM $MgCl_2$, 10mM dNTPs, 5pmoles each forward and reverse primers, 50-100 ng of template DNA and the volume was made up to 15 µl with milli Q water. The thermal-cycler conditions consisted of initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation (95°C for 45 sec), annealing (with temperature ranging between 51 and 61.5°C for 30 to 60 sec) and extension (72°C for 60 sec); and final extension (72°C for 10 min). The size of the PCR amplicon was verified in a horizontal submarine gel electrophoresis with 2 per cent agarose at 100 V. The amplified products were checked under a UV trans-illuminator and documented by a gel documentation system (Bio-Rad, USA). A total of 10 samples from CBJY and CBHF

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bulls were sequenced per primer set in an automated ABI PRISM 3730XL Genetic Analyzer.

The variations in sequences of CBJY and CBHF bulls were analysed using Editseq program of LASERGENE software (DNASTAR Inc., Madison, WI, USA-Version 2.1). The *Bos taurus* sequence was considered as the reference sequence. This software created the consensus sequence and highlighted the SNPs, which were verified manually by base calling using chromatogram. The SNP position was noted and the sequence variation was analysed among individuals within and between genetic groups. The SNPs thus detected were genotyped using the PCR-RFLP technique. The restriction site and the corresponding enzyme were identified using the online tool NEB cutter (*http:/ tools,neb.com/NEBuffer2*). The PCR product was digested with the restriction enzyme (*Mse* I) to genotype the individual bulls with respect to SNP. The digested products were checked in 2 per cent agarose gel along with 50 bp DNA ladder and the sequences with SNPs were subjected to translation VaxaSoftware (*http://rnat-rna-codons-toamino-acids-translato.software.informer.com/*) for the type of mutation and also to determine the protein structure.

Statistical analyses

The genotypes were assigned on the basis of restriction digestion pattern of the PCR products. The gene and genotype frequencies were calculated (Falconer and Mackey, 1996).

Basic statistics like mean and standard error were computed for semen volume, sperm concentration, mass activity, initial sperm motility, post-thaw motility and number of doses per ejaculate for CBJY and CBHF bulls. The data on different genotypes obtained through RFLP were subjected to least-squares analysis (Harvey, 1996). to estimate the association between SNPs with semen production data using the model, $Y_{ijk} = \mu + S_i + G_j + e_{ijk}$

Where, Y_{ijk} = semen production traits of k^{th} trait, i^{th} SNP and j^{th} genetic group; μ is the overall mean, S_i is the effect of i^{th} SNP (i = 1 to 3), G_j is the effect of j^{th} genetic group (j = 1 to 2) and e_{ijk} is the random error. The difference of means between semen production traits within genetic group and SNPs were tested for significance by applying Duncan's Multiple Range Test (Kramer, 1957).

RESULTS AND DISCUSSION

The sequence analysis revealed that the bovine Y-specific genes such as Testis-specific protein Y-encoded, Ubiquitin specific peptidase 9-Y-linked and DEAD box polypeptide 3-Y-chromosome were highly conserved and monomorphic across individuals among CBJY and CBHF bulls. However, the SRY gene had one mutation in its exon which was characterized by a G>T transversion at 641st position (i.e. 214th codon) among CBJY and CBHF bulls.

Genotyping of bovine SRY gene

The mutation in the exon of the SRY gene is represented schematically in Plate 1. The mutation was non-synonymous, replacing the amino acid cystein with phenylalanine in the protein assembly of *SRY* gene.

All three possible genotypes (GG, GT and TT) were observed in the samples screened with frequencies of 0.46, 0.20 and 0.34; and 0.46, 0.39 and 0.15 in CBJY and CBHF bulls respectively (Table 2). The frequency of the minor allele T was 0.44 in CBJY and 0.35 in CBHF bulls. The PCR-RFLP fragments digested with *MseI* showed the presence of GG, GT and TT genotypes (Plates 2 and 3).

Association of SNP with semen production parameters

Least-squares means for three SNP genotypes of SRY gene on semen volume, sperm concentration, mass activity, initial sperm motility, post-thaw motility and number of doses per ejaculate in CBJY and CBHF bulls are given in Table 3. In CBJY bulls, semen volume and sperm concentration were significantly ($P<0.05$) influenced by SNP genotypes of SRY gene. Among the three genotypes at 641 G>T, GG and GT genotypes were found to have higher semen volume and sperm concentration respectively. Whereas, this SNP did not influence the mass activity, initial sperm motility, post-thaw motility and number of doses per ejaculate in CBJY bulls. But, none of the semen production parameters in CBHF bulls was influenced by the SNP detected in SRY gene.

Sex determining region on Y-chromosome

The non-synonymous mutation, G>T transversion detected in the present study at 641st position of SRY gene on Y-chromosome, would replace the cystein with phenylalanine in the protein assembly. The minor allele (T) of the SNP was found distributed at higher frequency in both CBJY (0.44) and CBHF (0.35) bulls. In addition, this SNP had influenced the traits such as semen volume and sperm concentration in CBJY bulls. The bulls possessing the GG genotype produced higher semen volume, but lower sperm concentration as these two traits are negatively correlated. On the other hand comparatively, higher mean sperm concentration (1391.47 million per ml) was obtained in bulls which were carriers (GT, heterozygotes) for this mutation. None of the other traits *viz.* mass activity, initial sperm motility, post-thaw motility and number of doses per ejaculate was influenced by these genotypes in CBJY bulls. Moreover, lesser number of samples screened under CBHF bulls, could not generate a meaningful association with any of the semen production traits.

Critical perusal of literature revealed that the G>T tranversion at 641st position found in the present study is a new SNP detected in Jersey crossbred bulls as well as HF crossbred bulls. The exclusive presence of 'T' allele in the Indian crossbred populations indicated that the allele should be from the zebu cattle of Indian sub-continent. This fact is also supported by the higher frequency of this allele among the crossbreds. The study conducted by Mukhopadhyay et.al. (2011) revealed that no polymorphism was identified at the nucleotide level in SRY gene in Karan Fries bulls of India. The present investigation is distinguished from the earlier work (Verkarr et.al., 2013) in that a few sequence variants of the SRY gene were found across ox *(Bos taurus*), zebu (*Bos indicus*), gaur (*Bos gaurus*), yak (*Bos grunniens*), bison (*Bison bison*), water buffalo (*Bubalus bubalis*), African buffalo (*Syncerus caffer*) and Banteng (*Bos juvenicus*), but were stable in these species and that the segregation of species-specific Y-chromosomal variants has been complete. Even this study did not reveal the SNP at 641st position in zebu among 32 mutations detected across the species. Similarly, the variations at SRY gene at different nucleotide positions (61,122 and 187 bp) suggested that these variations would be used for species hybridization study in yak and their hybrids (Ramesha et.al., 2012).

The finding of fruitful association of this SNP with certain semen production parameters is unique in the sense that none of the earlier studies on SRY gene dealt any association of molecular markers with semen characteristics. Since,

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SRY gene is the most obvious target among the bovine Y-chromosome gene, this SNP (641 G>T) could be used as a candidate marker for the selection of bulls based on semen volume and sperm concentration.

Testis-specific protein Y-encoded

The greatest homology exhibited by the exonic regions of TSPY gene among CBJY and CBHF bulls screened in this study, is in agreement with the bovine TSPY gene expression where precise conservation was noticed, when compared to the human TSPY homolog (Vogel et.al., 2012). Contrary to our findings, a SNP was detected in fourth intron of bovine TSPY gene[10] which was reported to have no association with semen production traits (Mishra et.al., 2012).

Ubiquitin specific peptidase 9 -Y-linked

The USP9Y gene was found to be highly conserved across the bulls of both the genetic groups (CBJY and CBHF). This gene is one of the two genes located in the 'azoozpermia factor'- a (AZFa) region of Y-chromosome and thus cannot be considered as a major candidate gene involved in spermatogenesis and it might exert its effect in combination with other genes of the region (Vineeth and Malini, 2012). Rather than its variants or SNPs, major changes like the deletion or shortening of the USP9Y gene will only cause azoospermia or oligospermia or oligoasthenozoospermia in mammals (O'Flynn O' Brien et.al., 2010).

DEAD box polypeptide 3-Y-chromosome

The highly conserved nature of this gene among CBJY and CBHF bulls is in agreement with the earlier works in *Bos taurus* cattle (Hellborg and Ellegren, 2004).

In general, majority of the genes in the Y-chromosome are found to be involved in spermatogenesis and therefore bull fertility is considered to be a complex multigenic trait with heterogenous phenotypical expression of the concerned genes. Besides highly penetrant mutations in genes concerned involved with spermatogenesis, the interaction of factors, both genetic and environmental should also play an important role for phenotypic expression. Even though, mere mutations or polymorphisms in Y-specific genes, acting as fine-tuners or modulators of the efficiency of spermatogenesis, may not necessarily lead to clinically overt conditions, they are still relevant to the determination of spermatogenic potential of individual bull. Moreover, these variations are passed on to the offspring and possibly altering the expression of the trait in the subsequent generations. Thus, selection based on the SNP found in the SRY gene would yield better result with respect to breeding soundness of bulls. Further investigations are required to validate this Y-chromosome specific markers associated with andrological parameters in CBJY bulls.

Acknowledgement

The authors are thankful to the Tamil Nadu Veterinary and Animal Sciences University for the financial assistance provided to the Department of Animal Genetics and Breeding, Madras Veterinary College, Chennai to carry out this research.

Table 1. Designed primers and their annealing temperature for Y-chromosome specific genes

Primer name	Primer sequence (5' → 3')	Annealing temperature (°C)	Fragments size (bp)
1. Sex determining region on Y-Chromosome			
SRY	Forward : CGC AGT GCA GTC GTA TGC TTC TGC	61.5	736
	Reverse : AGC GCC TTT GTT AGC GAG AGT AAG G		
2. Testis specific protein Y-encoded			
TSPY I	Forward : CTA GTC GGT CCC ACA GGT TT	53	756
	Reverse : TTC TCC CCT TCC TCG TCC T		
TSPY II	Forward : ATC CTT GTT CCA CCC CAG AC	57.4	645
	Reverse : AGT TAA GGC TCC TGG TGT CC		
TSPY III	Forward : GAT GAG TGT GGG TGT GCA AG	59	686
	Reverse : ATT CAC CCT GCC TCT AGT CG		
TSPY IV	Forward : AGG ACA TGG TGC CTA GTG AC	59	606
	Reverse : TTC GGG TCT CTG CTC TTC TC		
3. Ubiquitin specific peptidase 9-Y linked			
USP9Y I	Forward : TCA CAG AAA ATT CCT CAT CAG C	58	429
	Reverse : GCC GGC AGT TTC AGA TAG TAA		

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USP9Y II	Forward: GAA AGC AGC CAT TCT ATA C Reverse: TAA CAA TGA AGG GTA GTC AC	51	852
USP9Y III	Forward: ACA CTG ACC ACC AGA AAT A Reverse: CAA CTG AAC TGA ACT GAA AG	55	452
USP9Y IV	Forward: GCC TTT TGT AAG GGA CAA Reverse: GGG TTG CAT ATC AAA AGC	53	335
USP9Y V	Forward: CTC AAT TCC TAG CAC AAA C Reverse: GCC AGA GAT GTG GTT AAA	53	643
USP9Y VI	Forward: GGA AAA TAC TCA GAG AGT GA Reverse: CCA CAT TGA ACA CAT AAC AC	52	406
4. DEAD box polypeptide 3-Y chromosome			
DDX3Y I	Forward : CGT TTA AAT ACA CCC CCA AG Reverse : AGG AAC CAG CAA AAG GAA GA	52.9	319
DDX3Y II	Forward : GTG TGT ATG ACT GGA ATT TAG GAC T Reverse : CAC ACT TGA AAA GAA CCA ACT AGT C	61	502
DDX3Y III	Forward : CCA GCT ATG TCT GGG AAA TGT G Reverse : AAG CAC AGA CGG GAG GGA AA	61.5	895
DDX3Y IV	Forward : CAG ATA ACC ACA GCT AAA TTG GAA G Reverse : ACA TTA GTC ACC AGT CTC TCC T	61	654
DDX3Y V	Forward : GAC AGA CAT TGA CAG ACA TT Reverse : ACT TTC CCT TCA CCG ACT CC	54	527
DDX3Y .VI	Forward : CAC AAA TCA TCC AGC ATT CTT CAC Reverse : GAC CAA TAT CAG CAC CAC CG	58.2	709
DDX3Y VII	Forward : TCT CCT TGG TTT TAG CCC CA Reverse : CCA CTC CAG TAC TCT TGC CT	61	402
DDX3Y VIII	Forward : TGG CTA TGT GAT CTG TAT GTG GT Reverse : ACA GGG TAC AGG CTA AAG GT	55	598
DDX3Y IX	Forward : TAC CTT TAG CCT GTA CCC TG Reverse : TGG CAC TTC TTG TTT GGC TT	61	840
DDX3Y X	Forward : TGG TTG CTT GAT GGT TTG ACA Reverse : GAA GCC TCC ATA GCC ACC TA	61	978
DDX3Y XI	Forward : CAG CAG CAG AGG ATT TGG TG Reverse : GGA GAA TCA CTA TGG GTA ATG CA	53	441

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Table 2. Gene and genotype frequencies of SNP of SRY gene in CBJY and CBHF bulls

SNP	Genotypes	Genotype frequency		Alleles	Allele frequency	
		CBJY (n=75)	CBHF (n=14)		CBJY (n=75)	CBHF (n=14)
641 G>T (214 – codon)	GG	0.46	0.46	G	0.56	0.65
	GT	0.20	0.39			
	TT	0.34	0.15	T	0.44	0.35

Table 3. Least-squares means of semen production traits for the three genotypes of SNP in SRY gene in CBJY and CBHF bulls

Genotypes	No. of bulls	Mean ± S.E.					
		Semen volume (ml)	Sperm concentration (millions per ml)	Mass activity (0 to 5 scale)	Initial sperm motility (per cent)	Post-thaw motility (per cent)	No. of doses per ejaculate
CBJY							
GG	35	3.89 [a] ± 0.17	1107.31[b] ± 39.81	3.35 ± 0.07	62.26 ± 2.77	49.20 ± 1.35	208.87 ± 10.57
GT	17	3.59[b] ± 0.20	1391.47[a] ± 111.76	3.56 ± 0.14	70.41 ± 2.72	51.29 ± 1.02	225.59 ± 15.86
TT	23	3.55[b] ± 0.16	1132.92[b] ± 31.03	3.33 ± 0.08	62.39 ± 3.59	50.18 ± 0.48	186.32 ± 11.53
CBHF							
GG	6	4.44 ± 0.42	867.67 ± 71.77	3.27 ± 0.16	50.83 ± 6.75	33.33 ± 7.50	152.50 ± 38.88
GT	6	3.36 ± 0.46	876.60 ± 73.62	3.14 ± 0.17	64.60 ± 7.40	49.80 ± 8.14	143.00 ± 42.59
TT	2	3.40 ± 0.73	1086.50 ± 124.31	3.55 ± 0.28	76.00 ± 11.70	51.50 ± 12.98	171.00 ± 67.33

Means with at least one common superscript within classes do not differ significantly (P>0.05).

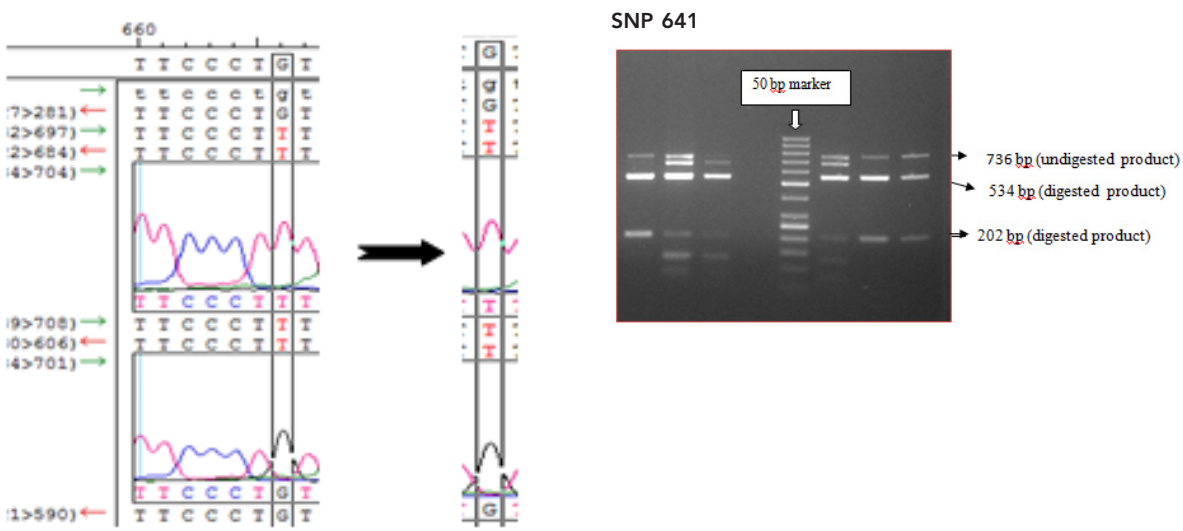

Plate 1 Chromatogram of SRY gene amplicon showing

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