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SNP Identification in Sperm Associated Antigen 11B Gene and Its Association with Sperm Quality Traits in Murrah Bulls

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ABSTRACT

Background: The beta-defensin family antigens present on sperm surface suggestively play pivotal role in sperm fertility by aiding in various steps of sperm maturation, motility, capacitation, immune recognition and sperm-oocyte interaction in female tract. It is imperative to explore genetic polymorphisms to build a better understanding of individual variation in male fertility. The experiment was designed to identify DNA polymorphism in sperm associated antigen 11B (SPAG11B) gene and to analyze association between genetic variants with sperm quality traits in buffalo bulls (Murrah) in ICAR-National Dairy Research Institute, Karnal.

Methods: Genomic DNA was extracted from hundred and thirty Murrah bulls. A 395 base pair region covering partial Intron 2, Exon 3 and Partial Intron 3 of bovine SPAG11 gene was amplified and genotyped using PCR-RFLP method. The PCR amplified product was purified, sequenced and further ClustalW analysis was done to align edited sequence with reported *Bos taurus* sequence (AC_000158.1). Gene and genotype frequencies, effective number of alleles, level of heterozygosity and polymorphic information content of various genotypes were estimated by standard procedure. Seminal parameters (Post thaw sperm motility, sperm viability, acrosomal integrity, HOST and abnormality) were estimated and statistical analysis was carried out.

Result: A novel SNP (G>A substitution) at 2266 base of the SPAG11B gene was identified by sequencing. The fragment of SPAG11 gene containing 395 base pairs was amplified using PCR and digested with restriction enzyme *i.e. MunI* which showed three distinct genotypes *viz*., AA (266 bp and 107 bp fragment), AG (307 bp, 266 bp and 107 bp) and GG (373 bp fragment). Least squares means of seminal parameters for the SNP was estimated and compared after correction for non-genetic factors. Between genotypes, significant differences were found only for acrosomal integrity and was highest in AG (74.22±0.72) followed by AA (72.6±0.89) and GG (71.12±0.97), respectively. The identified novel SNP of SPAG11 gene showed significant association with acrosome integrity with genotype AG being superior to other genotypes. However, an association cannot be established with other seminal parameters with this SNP, further studies are required in order to validate the impact of g.2266G>A on sperm quality traits in a large population.

Key words: *Mun*1, Murrah bulls, PCR-RFLP, Seminal parameters, SPAG11B.

INTRODUCTION

Artificial insemination (AI) is the most valuable reproductive bio-technique implemented in bovine reproduction, which greatly improved reproductive competence of the breeding bulls and thus achieved immense acceptability among farmers. For continuous improvement of productivity, availability of high genetic merit bulls with superior fertility is required for constant supply of quality frozen semen and their continuous replacement. The prime objective of frozen semen preparation is to harvest the maximal amount of high-quality sperm from genetically superior bulls for its widespread use in AI. Further, optimal fertility using frozen semen can be achieved by freezing of superior quality semen. However, improvement by direct selection in semen quality traits, including semen volume per ejaculate, sperm motility, sperm concentration and so on, is difficult; because of their considerably low heritability (Mathevon *et al*., 1998). Advancement in molecular genetics facilitated the use of polymorphisms in DNA as an aid called marker-assisted selection (MAS) for the selection of genetically superior animals. In goats and swine, various studies have been undertaken highlighting the significance of the candidate gene to be used as a marker for sperm quality traits (Wang *et al*., 2011; Huang *et al*., 2002; Wimmers *et al*., 2005; Lin *et al*., 2006). However, limited information on candidate marker genes in cattle bulls (Dai *et al*., 2009; Yang *et al*., 2011; Gorbani *et al*., 2009a,b) are available and rare studies were performed in buffalo bulls till date (Revanasiddu *et al*., 2019; Chandra *et al*., 2020).

In recent past, numerous research works has been undertaken to associate the number of candidate genes with

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bull fertility traits in many species. Sperm associated antigen 11 (SPAG11) gene among them requires special attention as SPAG11B (family β-defensin) is a gene encoding for several epididymis-specific, androgen-dependent, secretary proteins possibly involved in sperm maturation, acquiring motility, capacitation and sperm-egg interaction. The bovine SPAG11B gene is located on chromosome 27 (NCBI Accession no NC_000184.1). It performs both immune and reproductive functions in rats (Li *et al*., 2001; Zhou *et al*., 2004), humans (Yenugu *et al*., 2006a) and cattle (Avellar *et al*., 2007). In cattle SPAG11 gene is contained in β-defensin cluster. Due to its complex genomic organization and mRNA splicing pattern, SPAG11 is considered as remarkable member of human and bovine β-defensins (Schutte *et al*., 2002; Avellar *et al*., 2007). In addition to androgen regulation, other tissue-specific factors, hormones and/ or extracellular signals are differentially present in fetal and adult tissues, which are likely to influence the regulation of SPAG11 mRNAs in the bovines. Therefore, the SPAG11 gene suggestively is an excellent candidate for screening of reproductive performance associated mutations. Avellar *et al*. (2007) showed that, SPAG11C, SPAG11E and SPAG11U mRNA are located predominantly in the male reproductive tract, whereas SPAG11V and SPAG11W are restricted to testicular tissues in adult bulls. Both immune and reproductive functions are performed by SPAG11 isoforms in cattle (Avellar *et al*., 2007), rats (Li *et al*., 2001) and humans (Zhou *et al*., 2004). SPAG11 also plays a vital role in development of sperm motility (Zhou *et al*., 2004), a probable role in spermatogenesis and in antimicrobial protection during sperm passage through the reproductive tracts (Yenugu *et al*., 2006a,b); hence, variations in SPAG11 may also potentially contribute to the sperm quality traits. Moreover association of SPAG11B gene with conception rate in Murrah bulls (Deshmukh *et al*., 2019) also suggested the role of present gene in fertility related traits.

Single nucleotide polymorphism (SNP) in the SPAG11 gene has been searched for its association with sperm quality traits in cow bulls. There is a need to ensure whether such SNP have any significant association with semen quality parameters or not; so that it can be incorporated into the buffalo breeding programs in future. Thus current study was planned to explore presence of SNP/s in and around exon 3 of SPAG11B gene as well as its association with sperm quality traits in Murrah bulls.

MATERIALS AND METHODS

Resource population and semen analysis

Frozen semen samples from 130 Murrah bulls available at Artificial Breeding Research Centre (ABRC), ICAR-NDRI, Karnal, Haryana, India were included in the present study. For each bull, repeated measurements of sperm quality traits were performed. Three replication of frozen semen straws from each available season were used for each bull and thawed at 37°C for 30 sec and immediately evaluated for the post-thaw semen quality traits including post-thaw sperm motility, sperm viability, acrosome integrity, HOST and the percentage of abnormal sperm under light microscopy using standard protocols. The post-thaw cryopreserved sperm motility was viewed on a microscope by placing a drop of semen on to a pre-warmed (37°C) slide and overlaying it with a cover slip. The percentage of viable sperm and abnormalities were calculated by examining over 200 sperm cells per sample at a magnification of 100× oil immersion by staining with Eosin-Nigrosine methods. Acrosome integrity and HOST were observed at 100× oil immersion by Giemsa staining following Hancock (1952) and Correa and Zavos (1994) methods, respectively.

Genotyping of SNP

The SNP located on the exon 3 of SPAG11B gene was genotyped using sequencing and PCR-Restriction fragment length polymorphisms (PCR-RFLP). Genomic DNA was isolated from frozen-thawed semen samples using phenol-chloroform extraction method with slight modification of an addition of 25µl of 1M DTT /ml of semen before adding proteinase K. Forward primer 5′-GGCAGTTTCTTGGGGTCAAT -3′ and reverse primer 5′- GCACATCGCAGGTGCTTATT-3′ were used in PCR reaction to amplify target region (373 base pair) of SPAG11 gene consisting part of Intron 2, Exon 3 and part of Intron 3. The PCR reaction was carried out in 25 µl reaction volume containing 12.5 µl of DreamTaq™ Green PCR Master Mix (Fermentas, Life Sciences, USA), 1 µl each of upstream and downstream primers (10 pmol/µl) and template DNA. Standardized PCR reaction protocol included steps of initial denaturation for 3 min at 95°C, followed by 40 repeated cycles of denaturation for 30 seconds at 95°C, annealing for 35 seconds at 62°C and extension for 1 min at 72°C. At the end of the reaction a final extension for 5 min at 72°C was given. Twenty PCR products were then sequenced and their edited sequences were aligned by ClustalW method for identification of the SNPs and then it was subjected to restriction enzyme (RE) digestion. The preliminary selection of restriction enzymes to be used was done using NEB cutter V2.0 by submitting the *Bubalus bubalis* reference sequence obtained through sequencing of 20 samples. For PCR-RFLP analysis, amplified PCR products was digested using target specific '*MunI*' enzyme (Thermo Scientific, USA). The restriction digestion was done in a reaction volume of 25 µl containing 10 µl PCR product, 0.5 µl (5 U/µl) *MunI* enzyme, 2.0 µl buffer and 12.5 µl, nuclease free water, incubated for 10-12 hours at 37°C. The digested products were separated by 2.5-3% agarose gel electrophoresis (horizontal) and visualized by ethidium bromide staining (at 2 µl/100 ml of gel under UV light with a Gel Doc system (Bio-Rad).

Statistical analysis and association study

The gene and genotype frequencies, effective allele number, average heterozygosity and polymorphic information content of different genotypes were estimated by POPGENE version 1.32. For the statistical analysis of the sperm quality traits; 'Proc GLM' procedure of the SAS software package (Version 9.3)

was used. Initially, effects of non genetic factors *viz*. Period, season and age at freezing, were analyzed after that with the help of least squares constants, sperm quality data were adjusted for significant non genetic factors. Adjusted sperm quality traits were taken as dependent variable whereas genotypes of the fragment were considered as independent variables for the analysis of effect of genotypes.

RESULTS AND DISCUSSION

The SPAG11 gene in cattle is composed of six exons and five introns (spanning about 34 kb). It is located on chromosome 27q1.2 near the defensin gene cluster. The SPAG11 amplicon in Murrah buffalo as compared to *Bos taurus* contains three transversion, two transition and a deletion of 22 bp segment stretch. Direct sequencing and ClustalW alignment of the amplicon sequences from 20 Murrah bull samples revealed one novel SNP (g.2266G>A) in exon 3 of SPAG11 gene. For genotyping of the targeted SNP (g.2266G>A) in rest of the samples, a site specific restriction enzyme *i.e. MunI* was identified using NebCutter V2.0. Three distinct genotypes *viz*., AA (266 bp and 107 bp fragment) AG (307 bp, 266 bp and 107 bp) and GG (373 bp fragment) were obtained from restriction digestion of 395 bp fragment of SPAG11 gene followed by agarose gel electrophoresis (Fig 1). The SNP found was novel as homologous SNP for this site was not reported in buffalo or cattle. Chromatogram of SNPs at Position g.2266G>A in exon 3 of SPAG11 gene in Murrah Bulls is presented in (Fig 2).

The genotype frequencies of 0.2846, 0.4615 and 0.2538 were obtained for genotype AA, GA and GG, respectively and gene frequencies of 0.5154 and 0.4846 were obtained for allele A and G, respectively among the Murrah bulls under study. The gene frequency of allele A was found to be a little higher in population under study and the found difference in the genotype frequencies might be due to selection in the Murrah bulls since long. The Murrah population was found to be in Hardy Weinberg equilibrium ($p<0.05$) with respect to this SNP indicating that there was little selection pressure. The selection of Murrah buffalo bulls was done primarily for production improvement, however standard of minimum seminal parameters were set for cryopreservation that provided selection pressure though slight, on genes influencing seminal characteristics. The effective number of allele, Shannon's index, average heterozygosity, PIC and χ^2 values for the locus g:2266G>A were found to be 1.9981, 0.6927, 0.5015, 0.3748 and 0.8301 respectively. Polymorphic information content (PIC) for this genetic variant was found to be <0.5 indicating that the locus is moderately polymorphic in Murrah herd.

β-defensins are reported to be expressed in the male reproductive tract of mammals with clear region-specific patterns throughout the tract (Com*et al*. 2003; Jelinsky *et al*. 2007), suggesting at potential roles of β-defensins in reproduction. Defensin molecules modulate sperm surface–receptor presentation and interaction of spermatozoa's head to oocyte's zona at the time of fertilization. The SPAG11B homologus in rat known as Bin1b, is expressed in the proximal region of the epididymis and shows the ability to initiate the sperm maturation process by inducing progressive motility in previously immotile spermatozoa (Zhou *et al*. 2004). Further work has also shown that spermatozoa coated with β-defensin 126 are protected from immune recognition and clearance by the female immune system in macaque (Yudin *et al*. 2005). Interestingly, single-nucleotide polymorphisms (SNPs) in the gene encoding this peptide (SPAG11) have been associated with sperm volume and motility traits in cattle (Liu *et al*. 2011). Thus the polymorphisms in this gene hold potential as marker with respect to bull fertility.

The effects of the locus (g.2266G>A) on semen parameters namely post-thaw sperm motility, the percentage of abnormal spermatozoa, sperm viability, acrosome integrity and HOST of 130 Murrah bulls are summarized in (Table 1). The g.2266G>A SNP was found significantly associated with the acrosome integrity. The role of SPAG11 in gametogenesis and sperm maturation has been laid down by evidences of their major expression in male reproductive organs as well as higher expression in adult testes, besides confirming presence of SNPs in SPAG11 in monkeys by Avellar *et al*. (2007). As shown in Table 1, the bulls with the

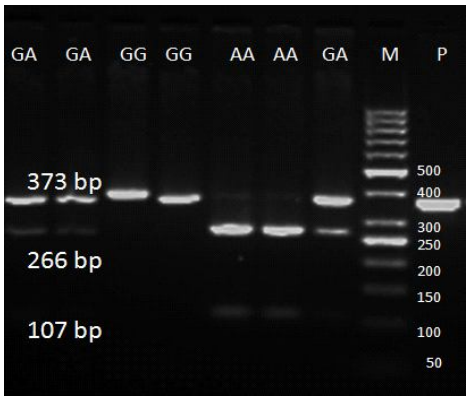

Fig 1: PCR-RFLP of 373 base pair fragment of SPAG11 gene in Murrah bulls using *MunI* restriction enzyme.
Lane 1-8: RE patterns, Lane M: 50 bp ladder, Lane P: PCR Product.

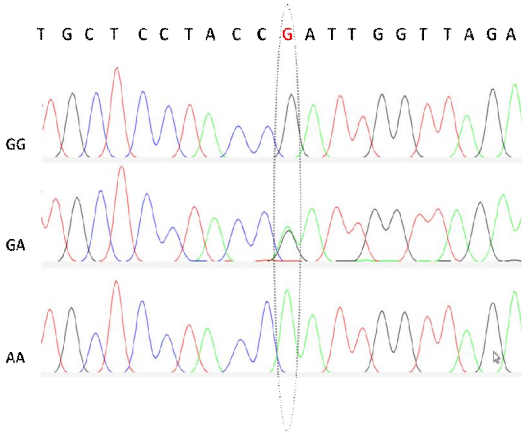

Fig 2: Chromatogram of SNPs at position 289 G>A of SPAG11 gene in Murrah bulls.

Table 1: Least squares means of novel SNP (2266G>A) of SPAG11 gene for different seminal parameters in Murrah bulls.

SNPs		PTM (%)	Abnormality (%)	Viability (%)	Acrosome integrity (%)	HOST (%)
289G>A	AA	54.71^{a}±0.93	8.16^{a}±0.32	65.02^{a}±1.10	72.6^{ab}±0.89	52.51^{a}±0.62
	AG	56.52^{a}±0.74	8.19^{a}±0.25	66.08^{a}±0.88	74.22^{b}±0.72	52.39^{a}±0.49
	GG	55.86^{a}±1.00	7.75^{a}±0.34	66.68^{a}±1.19	71.12^{a}±0.97	51.25^{a}±0.67

genotypes AG and AA have significantly higher acrosomal integrity (P<0.05) than those of genotype GG. However,the SNP showed no significant association to any of the other semen quality parameters. In Chinese Holstein bulls, Liu *et al*. (2011) has reported 6 SNPs in SPAG11 gene as 3 completely linked groups and found association with sperm motility that augments our findings. Our findings also indicate that SPAG11 might have an important role in acrosomal reaction, thus genetic variations in the SPAG11 gene of the buffalo bulls may be important for fertility.

CONCLUSION

The semen qualitative parameters are polygenic traits, thus they are also affected by environmental components to a considerable amount. The differences among genotypes may be thus either minute or masked by the exterior factors including cryopreservation and subsequent thawing of the semen. Statistical analysis yielded significant association between the identified genotypes with acrosomal integrity. Although, there is lack of association with other seminal parameters, studies are further required to investigate such more polymorphisms in this region and to understand the complicated genetic mechanisms of bull fertility traits in a larger number of buffalo bulls. Finally, discovery of regulatory and non-synonymous polymorphisms in SPAG11 gene could provide useful markers for the selection of bulls with superior fertility. These SNPs could become important tools with respect to improved buffalo breeding.

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REFERENCES

Avellar, M.C.W., Honda, L., Hamil, K.G., Radhakrishnan, Y., Yenugu, S., Grossman, G., Petrusz, P., French, F.S., Hall, S.H. (2007). Novel aspects of the sperm-associated antigen 11 (SPAG11) gene organization and expression in cattle (*Bos taurus*). Biology of Reproduction. 76: 1103-1116.

Chandra, S., Das, D.N., Kannegundla, U., Kour, R.J., Ramesha, K.P., Nath, S., Kataktalware, M.A. (2020). Polymorphism in inhibin alpha gene and its association with semen quality traits in Murrah bulls. Indian Journal of Animal Research. 54: 399-404.

Com, E., Evrard, B., Roepstorff, P., Aubry, F., Pineau, C. (2003). New insights into the rat spermatogonial proteome: Identification of 156 additional proteins. Molecular and Cellular Proteomics. 2: 248-261.

Correa, J.R. and Zavos, P.M. (1994). The hypoosmotic swelling test: Its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. Theriogenology. 42: 351-360.

Dai, L., Zhao, Z., Zhao, R., Xiao, S., Jiang, H., Yue, X., Li, X., Gao, Y., Liu, J., Zhang, J. (2009). Effects of novel single nucleotide polymorphisms of the FSH β-subunit gene on semen quality and fertility in bulls. Animal Reproduction Science. 114: 14-22.

Deshmukh, B., Verma, A. Gupta, I.D., Kashyap, N. and Behera, R. (2019). Association study of single nucleotide polymorphism in exon 3 of SPAG11B gene with conception rate in Murrah bulls. International Journal of Current Microbiology and Applied Sciences. 8: 1535-1541.

Gorbani, A., VaezTorshizi, R., Bonyadi, M., Amirinia, C. (2009a). Restriction fragment length polymorphism of bovine growth hormone gene intron 3 and its association with testis biometry traits in Iranian Holstein bull. African Journal of Microbiology Research. 3: 809-814.

Gorbani, A., VaezTorshizi, R., Bonyadi, M., Amirinia, C. (2009b). AMspI PCR-RFLP within bovine growth hormone gene and its association with sperm quality traits in Iranian Holstein bulls. African Journal of Biotechnology. 8: 4811-4816.

Hancock, J.L. (1952). The morphology of bull spermatozoa. Journal of Experimental Biology. 29:445-453.

Huang, S.Y., Chen, M.Y., Lin, E.C., Tsou, H.L., Kuo, Y.H., Ju, C.C., Lee, W.C. (2002). Effects of single nucleotide polymorphisms in the 5 -flanking region of heat shock protein 70.2 gene on semen quality in boars. Animal Reproduction Science. 70: 99-109.

Jelinsky, S.A., Turner, T.T., Bang, H.J., Finger, J.N., Solarz, M.K., Wilson, E., Brown, E.L., Kopf, G.S., Johnston, D.S. (2007). The rat epididymal transcriptome: Comparison of segmental gene expression in the rat and mouse epididymides. Biology of Reproduction. 76: 561-570.

Li, P., Chan, H.C., He, B., So, S.C., Chung, Y.W., Shang, Q., Zhang, Y.D., Zhang, Y.L. (2001). An antimicrobial peptide gene found in the male reproductive system of rats. Science. 291: 1783-1785.

Lin, C.L., Ponsuksili, S., Tholen, E., Jennen, D.G., Schellander, K., Wimmers, K. (2006). Candidate gene markers for sperm quality and fertility of boar. Animal Reproduction Science. 92: 349-363.

Liu, X., Ju, Z., Wang, L., Zhang, Y., Huang, J., Li, Q., Li , J., Zhong, J., An, L., Wang, C. (2011). Six novel single nucleotide polymorphisms in SPAG11 gene and their association with sperm quality traits in Chinese Holstein bulls. Animal Reproduction Science. 129: 14 -21.

Mathevon, M., Buhr, M.M. and Dekkers, J.C.M. (1998). Environmental, management and genetic factors affecting semen production in Holstein bulls. Journal of Dairy Science. 81: 3321-3330.

Revanasiddu, D., Ramesha, K.P., Kour, R.J., Kumar, A.M., Divya, P., Kataktalware, M.A., Basavaraju, M., Das, D.N., Anandkumar, N. and Sapna, N. (2019). Genetic variants in male specific region (MSY) of ZNF280BY gene and their association with semen quality traits in Murrah buffalo bulls. Indian Journal of Animal Research. 53: 1135-1139.

Schutte, B.C., Mitros, J.P., Bartlett, J.A., Walters, J.D., Jia, H.P., Welsh, M.J., Casavant, T.L., McCray Jr., P.B. (2002). Discovery of Five Conserved Beta Defensin Gene Clusters using a Computational Search Strategy. Proceedings of the National Academy of Sciences of the United States of America. 99: 2129-2133.

Wang, C.F., Liu, M., Li, Q.L., Ju, Z.H., Huang, J.M., Li, J.B., Zhong, J.F. (2011). Three novel single-nucleotide polymorphisms of MBL1 gene in Chinese native cattle and their associations with milk performance traits. Veterinary Immunology and Immunopathology. 139: 229-236.

Wimmers, K., Lin, C.L., Tholen, E., Jennen, D.G., Schellander, K., Ponsuksili, S. (2005). Polymorphisms in candidate genes as markers for sperm quality and boar fertility. Animal Genetics. 36: 152-155.

Yang, W.C., Tang, K.Q., Yu, J.N., Zhang, C.Y., Zhang, X.X., Yang, L.G. (2011). Effects of MboII and BspMI polymorphisms in the gonadotropin releasing hormone receptor (GnRHR) gene on sperm quality in Holstein bulls. Molecular Biology and Reproduction. 38: 3411-3415.

Yenugu, S., Hamil, K.G., French, F.S. and Hall, S.H. (2006a). Antimicrobial actions of human and macaque sperm associated antigen (SPAG) 11 isoforms: Influence of the N-terminal peptide. Molecular and Cellular Biochemistry. 284: 25-37.

Yenugu, S., Hamil, K.G., Grossman, G., Petrusz, P., French, F.S. and Hall, S.H. (2006b). Identification, cloning and functional characterization of novel sperm associated antigen 11 (SPAG11) isoforms in the rat. Reproduction Biology Endocrinology. 4: 23.

Yudin, A.I., Tollner, T.L., Li, M.W., Treece, C.A., Overstreet, J.W. and Cherr, G.N. (2003). ESP13.2, a member of the β-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. Biology of Reproduction. 69: 1118-1128.

Yudin, A.I., Generao, S., Tollner, T., Treece, C., Overstreet, J., Cherr, G. (2005). DEFB126 on the cell surface protects sperm from immunorecognition and binding of anti-sperm antibodies. Biology of Reproduction. 73: 1243-1252.

Zhou, C.X., Zhang, Y.L., Xiao, L., Zheng, M., Leung, K.M., Chan, M.Y., Lo, P.S., Tsang, L.L., Wong, H.Y., Ho, L.S., Chung, Y.W., Chan, H.C. (2004). An epididymis specific β-defensin is important for the initiation of sperm maturation. Nature Cell Biology. 6: 458-464.