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Association Study of Single Nucleotide Polymorphism in Exon 3 of SPAG11B Gene with Conception Rate in Murrah Bulls

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A B S T R A C T

Present study was conducted with the objectives to identify single nucleotide polymorphism in sperm associated antigen 11 B(SPAG11B) gene and to analyze association between identified polymorphism with conception rate in Murrah bulls in ICAR-National Dairy Research Institute (NDRI) herd, Karnal. A 373 base pair region covering partial intron 2, exon 3 and partial intron3 of bovine SPAG11 gene was amplified using genomic DNA extracted from eighty six Murrah bulls and genotyped using sequencing and polymerase chain reaction- restriction fragment length polymorphisms (PCR-RFLP) methods. Alignment of edited sequence of Murrah buffalo with reported *Bos taurus* sequence (AC_000158.1) was done with ClustalW analysis. Gene and genotype frequencies, effective allele number, average heterozygosity and polymorphic information content of different genotypes were estimated by POPGENE version 1.32 (University of Alberta, Canada). Statistical analysis for Conception rate of Murrah bulls under study was carried out by SAS Version 9.3(SAS Institute Inc., Cary, NC, USA). The 373 bp fragment of SPAG11 gene was amplified using PCR and a novel SNP of G to A substitution at 2266 base of the SPAG11B gene was identified by sequencing. The amplicon was further digested with SNP-specific *MunI* restriction enzyme that showed three distinct genotypes viz., AA (266 bp and 107 bp fragment), GA (307 bp, 266 bp and 107 bp) and GG (373 bp fragment). Least square means of conception rate for the SNP was estimated and compared after correction for non-genetic factors. The identified novel SNP of SPAG11 gene showed non-significant association with conception rate. However, there is a lack of association, further studies have to be undertaken in a large population in order to validate the impact of g.2266G>A on conception rate in Murrah bulls.

Introduction

Buffalo is the principal dairy animal in the developing Asian countries and is the mainstay of the Indian dairy industry. India resides world's largest buffalo population as 108.7 million. In India, the buffalo population currently producing 70.44 million tons of milk, contributes 51.16 percent of the total milk despite they account for only 36.28 percent of total bovine population (Anonymous, 2015). India is the native tract of the world's premium buffalo breeds, although a huge scope of further improvement in productivity still remains. A higher selection intensity for faster genetic growth

and success of AI technique calls for lesser bulls to cover a large breed able population, increasing the impact of bull fertility on overall reproductive performance. Continuous improvement of productivity demands higher availability of superior bulls with high genetic merit and fertility together (Lucy, 2001). An important indicator of fertility is conception rate (CR), which is defined as the percentage of females diagnosed as pregnant by conventional means (rectal palpation, ultrasonography, hormonal assay) at some specified interval after AI (e.g. 60 days).

Progress in molecular genetic techniques has enabled the application of DNA polymorphism as an aid to the selection of animals through what is known as marker-assisted selection (MAS). Recently, many reports regarding the use of the candidate gene as a marker for semen quality from swine (Huang *et al.,* 2002; Wimmers *et al.,* 2005; Lin *et al.,* 2006) and goats (Wang *et al.,* 2011a) have been conducted. However, few studies have reported on candidate marker genes in cow bulls (Dai *et al.,* 2009; Yang *et al.,* 2011; Gorbani *et al.,* 2009a, b) and rare studies performed in buffalo bulls.

There are a number of candidate genes associated with sperm quality of which Sperm Associated Antigen 11 (SPAG11) gene requires special attention. SPAG11 (family β-defensin) is a gene which encodes several androgen-dependent, epididymis-specific secretary proteins which are involved in sperm maturation, acquiring motility, capacitation, and sperm-egg interaction. In cattle, the sperm-associated antigen 11 (SPAG11) gene is placed in β-defensins cluster and is a unique member among the human and bovine β-defensins due to its complex genomic organization and mRNA splicing pattern (Schutte *et al.,* 2002; Avellar *et al.,* 2007). The SPAG11 gene, also known as epididymal protein 2 (EP2) in monkeys, human epididymis 2 (HE2) in humans, and Bin-1b in rats, performs both immune and reproductive functions in humans (Yenugu *et al.,* 2006), rats (Li *et al.,* 2001; Zhou *et al.,* 2004) and cattle (Avellar *et al.,* 2007). Avellar *et al.,* (2007) showed that in adult bulls, SPAG11C, SPAG11E, and SPAG11U mRNA are predominantly located in the male reproductive tract, whereas SPAG11V and SPAG11W are confined to testicular tissues. Also, SPAG11 plays a critical role in sperm maturation such as in the development of sperm motility (Zhou *et al.,* 2004), a possible role in spermatogenesis, and in antimicrobial protection during sperm passage through the male and female tracts (Yenugu *et al.,* 2006). As semen quality traits are crucial factors affecting conception rate for bulls; the SPAG11 gene appears to be a good candidate for mutations associated with bull's reproductive performance. The current study was undertaken to identify SNPs in SPAG11 gene which affects bull fertility in Murrah bulls that can be incorporated into the future buffalo breeding programs in India.

Materials and Methods

Ethical approval

The present experiment was approved by the Institutional Animal Ethics Committee of the ICAR-National Dairy Research Institute, Karnal, Haryana.

Place of study and resource population

The geographical location of N.D.R.I. Livestock Farm is at an altitude of 250 meters above the mean sea level in the Indo-Gangetic alluvial plains on 29.68°N latitude and 76.98°E longitude. The climate of the farm is subtropical in nature. Each year was sub-classified into four major seasons viz., winter (December to March), summer (April to June), rainy (July to September), and autumn

(October to November), depending on prevalent meteorological factors as recorded in Central Soil Salinity Research Institute, Karnal (Singh, 1983). The animals in the study consisted of total86 Murrah bulls having semen samples and records of AI maintained at Artificial Breeding Research Centre, ICAR-National Dairy Research Institute, Karnal, India.

Genotyping of SNP

Genomic DNA was isolated from frozen semen samples of all the bulls using phenol-chloroform extraction method with an addition of 25µl of 1M DTT /ml of semen prior to addition of proteinase K. Primers 5'-GGCAGTTTCTTGGGGTCAAT -3' (Forward) and 5'- GCACATCG CAGG TGCTTATT -3' (Reverse) were used for the PCR reaction for amplifying the 373 base pair covering partial intron 2, exon 3 and partial intron 3 region of bovine SPAG11 gene (Figure 1). The PCR reaction was carried out in a final volume of25 µl reaction mixture containing 12.5 µl of Dream Taq^{TM} Green PCR Master Mix (Fermentas, Life Sciences, USA), 9.5 µL nuclease free water and1 µl each of template DNA, forward and reverse primers (10 pmol/µl). PCR reaction profile consisted of initial denaturation at 95°C for 3 min, followed by 40repeated cycles of denaturation at 95° for 30 sec, annealing at 62°C for35 sec and extension at 72°C for 1 min followed by final extension for5 min at 72°C. Twenty amplicons were sequenced and aligned by ClustalW for identification of the SNPs and then it was subjected to restriction enzyme (RE) digestion. For PCR-RFLP analysis, amplified PCR products of exon 3 was digested with site specific *MunI* enzyme (Thermo Scientific, USA). The digestion was done in a final volume of 25 µL containing 0.5 µL (5 U/µL) restriction enzyme, 2.0 µL buffer, 12.5 µL nuclease free water and 10 µL PCR product, incubated in 37 °C for 10-12 hrs. Separation of digested products was done by 2.5–3 % agarose gel (with added ethidium bromide at 2 µL/100 mL) horizontal electrophoresis and visualized with the help of gel doc system (Bio-Rad).

Recording of data

The complete AI records of total 86 Murrah bulls under study for a period of 35 years (1980 to 2014) and its success was obtained from records of reproductive history cum pedigree sheets maintained at Livestock Record Unit of Dairy Cattle Breeding Division, ICAR-National Dairy Research Institute, Karnal, India. The records were used for estimation of conception rate for Murrah bulls.

Statistical analysis and association study

The genetic polymorphism parameters like gene and genotype frequencies, effective number of alleles, average heterozygosity and polymorphism information content of different genotypes were estimated using POPGENE (version 1.32) software.

For the statistical analysis of the conception rate; the SAS package (Version 9.3) was used. Non-genetic factors (NGF) were classified into subgroups as per Table 1, analyzed for effects of NGF on conception rate in Murrah bulls and then data were adjusted for the significant NGF i.e. season and period of AI using least square constants. Corrected data were considered as dependent variable whereas genotypes of the animals were taken as independent variables for the analysis of effect of genotypes. Following model was used for the analysis of effects of genotypes; $Y_{lm} = \mu + G_l + e_{lm}$ where, Y_{lm} is the corrected observation of m^{th} bull having l^{th} genotype, µ is the overall mean, G_l is the fixed effect of l^{th} genotype and e_{lm} is the random error.

Results and Discussion

The bovine SPAG11 gene is located on chromosome 27q1.2 and is composed of six exons (3 coding exons) and five introns, spanning about 34 kb. Recent studies conducted on cattle semen confirm that several sperm surface proteins can be used as markers for male fertility (Moura *et al.*, 2006), and SPAG11 is one of them. Thus the polymorphisms in this gene contain potential as candidates for the male fertility.

Total five nucleotide changes (3 transversion and 2 transition) and a deletion of 22 bp segment were found when sequence of Murrah buffalo was compared with *Bos taurus* and one SNP (g.2266G>A) in exon 3 was detected from 20samplesthrough direct sequencing and ClustalW alignment of the sequences. For SNP g.2266G>A, a site specific restriction enzyme *MunI* was identified. In Murrah bulls, the 373 bp fragment of SPAG11 upon restriction digestion, yielded three distinct genotypes viz., AA (266 bp and 107 bp fragment),GA (307 bp, 266 bp and 107 bp) and GG (373 bp fragment) (Figure 2). The SNP found was novel and no homologous SNP for this site was reported in buffalo or cattle. However 6 SNPs have been reported in exonic region of SPAG11 gene in Chinese Holstein bulls by Liu *et al.*, (2011) as 3 completely linked groups.

The genotype frequencies for genotype AA, GA and GG were 0.2847, 0.4615 and 0.2538, respectively and gene frequencies of allele A and G were0.5154 and 0.4846, respectively in the Murrah bulls. Allele A was marginally predominant in the present population and the difference in the genotype frequencies might be due to selection in the Murrah bulls. The population under study was found to be in Hardy Weinberg equilibrium ($p<0.05$) with respect to this SNP indicating that there was negligible selection pressure on the locus. 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.

A number of possible interpretations support the speculation that genetic variations in the SPAG11 gene of the buffalo bulls may be important for fertility. Numerous studies found isoforms of SPAG11 performing both immune and reproductive functions in species like rats (Li *et al.*, 2001), humans (Zhou *et al.*, 2004) and cattle (Avellar *et al.*, 2007). Specific affinity of SPAG11 isoforms in mRNA expression to majorly or exclusively in male reproductive tract (Avellar *et al.*, 2007) is in support to the suggestion. Further, SPAG11 plays a critical role in the development of sperm motility (Zhou *et al.*, 2004), spermatogenesis and antimicrobial protection of spermatozoa (Yenugu *et al.*, 2006).

In the present study, 5439 AI records of 86 Murrah bulls spanning over 35 years with season, period, parity and female AI number as NGFs, were utilized for calculation of conception rate per bull. The genotypes AA and AG have higher mean conception rate as compared to genotype GG but the differences were not significant. Effect of the locus (g.2266G>A) on conception rate of 86Murrah bulls are summarized in Table 2. The results of association study for the locus were confirmed by chi-square analysis with respect to distribution of animals in different fertility groups viz. high, medium and low with conception rate >42%, 35-42% and <35%, respectively. Animals with GA genotype were fewer in low fertility group whereas animals with genotype GG were found least in high fertility groups (Table 3).

Table.1 Classification of non-genetic factors (season of AI, periods of AI, parity and female A.I. no) for conception rate in Murrah bulls

S. No.	Season of AI	Period	Parity	Female AI no
1	Summer (Apr-Jun)	1980 - 1996	First	First AI after calving
2	Rainy (Jul-Sept)	1997 - 2002	Second	Second AI after calving
3	Autumn (Oct-Nov)	2003 - 2008	Third	Third AI after calving
4	Winter (Dec-Mar)	2009 - 2014	Fourth	Fourth AI after calving
5	-	-	Fifth and above	Fifth and more AI after calving

Table.2 ANOVA of conception rate for genotypes of SPAG11 gene

Source	Degree of Freedom	Sum of Squares	Mean Sum of Square	F value
289 G>A	2	48.69	24.34	0.78

Table.3 Least square means ± S.E. of conception rate for genotypes of SPAG11 gene

Locus	Genotypes	No. of bulls	CR
289 G>A	AA	25	39.85±1.85
	GA	41	38.60±1.44
	GG	20	36.32±2.06

Table.4 Chi-square analysis of genetic variants of SPAG11 gene and bull fertility level in Murrah bulls

SNP	Genotype	Fertility levels						Total	χ^2 value	P Value
		High >42%		Medium 35-42%		Low<35%				
289 G>A	AA	9	10.47	8	9.30	8	9.30	25	2.21	0.70
	GA	15	17.44	15	17.44	11	12.79	41		
	GG	4	4.65	8	9.30	8	9.30	20		

Fig.1 PCR amplification of 373 base pair fragment covering partial intron 2, exon 3 and partial intron 3 region of SPAG11 gene in Murrah Buffalo

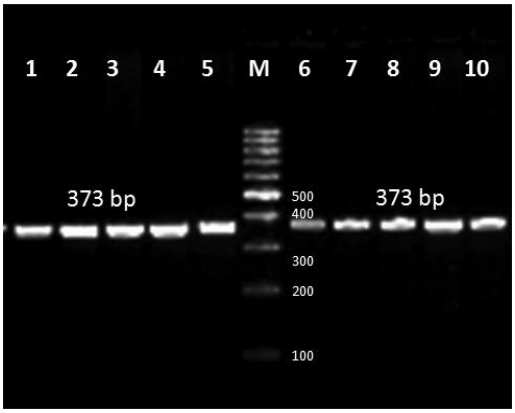
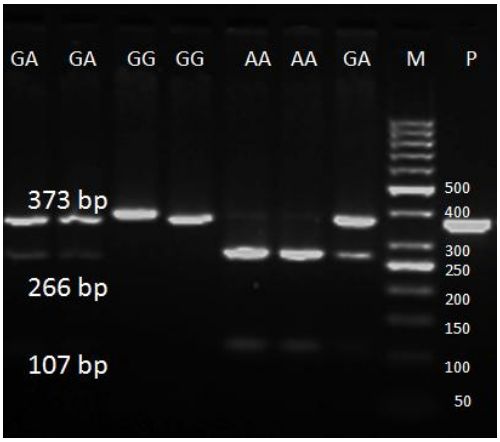


Fig.2 PCR-RFLP of 373 base pair fragment of SPAG11 gene in Murrah Buffalo



However χ^2 analysis indicated that the genotypes and fertility groups were statistically independent (Table 4).

Till date, no any report on association of SNPs in SPAG11 gene with conception rate in Murrah bulls to compare and contrast with the findings of present study. Therefore, findings of the present study are compared with the available sporadic reports on cattle. Out of the six SNPs of Bovine SPAG11 gene identified by Liu *et al.,* (2011),two SNPs were significantly correlated with the sperm quality traits i.e. sperm concentrations and fresh sperm motility while one SNP was significantly correlated with post-thaw sperm motility and percentage of abnormal spermatozoa ($P < 0.01$). However the SNPs were identified in Chinese Holstein Cattle and were non-homologous to the SNPs identified in Murrah bulls. Thus even if the SNPs detected in the exon 3 of SPAG11 gene show no statistical relation to conception rate in Murrah bulls, considering the importance of the gene, further exploration of the whole gene for identification of SNPs affecting fertility is called for, to ensure betterment of male fertility in Murrah bulls.

The selection of the SPAG11 polymorphism in a breeding program may improve male fertility that may further result in higher success rate of Artificial Inseminations leading to better reproductive efficiency of the herd. Statistical analysis yielded no significant association between the genotypes with conception rate. Although, there is lack of association, further studies are required to investigate more such polymorphisms and to understand the complex genetic mechanisms of bull fertility traits in a larger number of buffalo bulls.

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