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A SNP (g.358A > T) at intronic region of CD9 molecule of crossbred bulls may associate with spermatozoal motility

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ABSTRACT

The surface expression of CD9 (cluster-of-differentiation antigen-9) in sperms of certain mammalian species has been attributed to its fusion with the egg and thereby dictating the fertility of species. In the present study, we investigated the association of CD9 with crossbred bull sperm quality and quantity trait was analyzed using a total of 96 Frieswal (HF × Sahiwal) crossbred. A single nucleotide polymorphism (g.358A > T) in intron 6 was significantly associated with sperm concentration ($P < 0.05$) and motility percentage ($P < 0.01$). mRNA was extracted from good (progressive motility >50%) and motility impaired (progressive motility <50%) bull semen. The mRNA expression and seminal plasma protein concentration of CD9 was significantly ($P < 0.05$) higher among good quality bull semen than motility impaired ones. Our results thus may indicate that, mutation in the intronic region may be responsible for the instability of RNA and the subsequent functional protein expression.

1. Introduction

CD9 (cluster-of-differentiation antigen-9), a tetraspanin family protein is known to be widely present on cell membrane. It can mediate the process of signal transduction which is responsible for playing roles in cell development, activation, motility as well as growth. CD9 is mainly located at the microvillar membrane of oocytes and helps in maintaining the normal shape of oocyte microvilli. CD9 also plays important task for regulating the multifunctional activities during sperm oocytes interaction (Charrin et al., 2003; Hemler, 2003; Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000).

Certain studies have been undertaken using mouse and boar spermatogonal model. The expression of CD9 on mouse sperm was reported by Barraud-Lange et al. (2012). Significant expression of CD9 have been identified in Leydig cells, Sertoli cells and germ cells of boar testis, the epithelial cells of epididymis, vas deferens and prostrate glands as well as in spermatozoa within the lumen of epididymis (Kaewmala et al., 2011). In the same experimental model, Kaewmala et al. (2011) also investigated that SNP at CD9 gene that was associated with boar sperm quality and fertility traits. Recently, Cupperová et al. (2014) experimentally shown that CD9 molecules are expressed in different reproductive tissues as well as bull semen. As the phenotypic selection for reproductive parameters can only be carried out after puberty, genetic marker based selection could be tool of interest to improve bull fertility.

The study of the association of CD9 with sperm quality and quantity traits in close approximation with a functional approach is key elements to identify its applicability as a bio marker. Therefore, the objective was to investigate whether a SNP (earlier identified by Kaewmala et al., 2011) at CD9 gene is associated with bull semen quality or not. The present study also aimed to analyze the transcript abundance of CD9 between good and poor quality bull semen.

2. Materials and methods

2.1. Experimental animals and data collection

A total of 96 mature Frieswal (HF × Sahiwal) bulls in Bull Rearing Unit, Central Institute for Research on Cattle, Meerut, UP, India were included in the present study. Semen was collected using artificial vagina from each bull. The ejaculates were assessed for normal quality of semen and then categorized as good sperm motility (>50% progressively motile sperm) and the sperm motility impaired groups (<50% progressively motile sperm). The criteria used for classification was low sperm progressive motility that was supplemented with the hypoosmotic swelling response (HOST). Immediately after collection, the ejaculates were stored at 37 °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 fresh semen was then diluted

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with glycerol-egg yolk-citrate, processed and cryo-preserved. After storage in liquid nitrogen for 1–2 days, two straws were randomly obtained from each ejaculate and thawed at 37 °C for 60 s and immediately evaluated for post-thaw motility (PTM) (%) with light microscopy.

2.2. Isolation of sperm DNA, primer designing and PCR conditions

Genomic DNA was extracted from the total 96 bull semen samples using GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich, USA). The DNA samples were dissolved in elution buffer (supplied with kit) and stored at −20 °C for future use. A set of primers were synthesized (Table 1) to amplify a 563 base pair fragment coding intron 6 region of bovine of CD9 gene. The PCR was performed in a 25 μl reaction mixture containing 1× PCR buffer (Sigma-Aldrich, USA), 1.5 mM $MgCl_2$ (Sigma-Aldrich, USA), 200 μM dNTPs (Sigma), 10 pmol of each primers, 1 U Taq DNA polymerase (Sigma-Aldrich, USA) and 50 ng of genomic DNA as template. The cycling protocol was initially denatured for 5 min at 95 °C followed by 35 cycles (94 °C for 50 s, 55 °C annealing for and 40 s, 72 °C for 45 s), with a final extension at 72 °C for 10 min. Amplicons were visualized by electrophoresis in 1.5–2% agarose gels containing 500 ng/ml of ethidium bromide in 1× TAE buffer with a 100-bp ladder as a molecular weight marker for confirmation of the length of the PCR products.

2.3. SSCP analysis, cloning and sequencing

For SSCP analysis, several factors were tested for this amplicon in order to optimize the amount of PCR products, acrylamide concentration, percentage of cross-linking glycerol, running time and voltage. PCR products (10 μl) were mixed with 10 μl denaturing dye (95% formamide; 20 Mm EDTA, pH 8.0; 0.025% bromophenol blue; 0.025% xylene cyanol). The samples were then denatured by heating for 5 min at 95 °C, after which they are chilled on ice for 5 min and loaded on to a non-denaturing 15% polyacrylamide gel (45:1 acrylamide:bisacrylamide) in 1× TBE buffer. Electrophoresis was carried out at 4 °C, 100 V for 16–18 h. The gels were subsequently fixed in 70% ethanol, stained with 0.2% silver nitrate ($Ag\ NO_3$) and visualized with 3% sodium carbonate ($Na_2\ CO_3$). Different genotypes obtained for both the locus were subjected for TA cloning using pTZ57R plasmid (InsTAclone PCR Cloning kit, Fermentas). The positive clones were confirmed by restriction enzyme analysis. Positive clone from each genotype were subjected to DNA sequencing.

2.4. Real time PCR based differential expression of CD9 gene

Fresh semen samples were collected from categorized crossbred Frieswal bulls into normal (good) and impaired groups (poor) according to the spermatozoa progressive motility (more than 50% considered as good quality). The concentration of sperm was estimated by using a photometer (Accucell, IMV-France). To rule out the possibility of spermatozoa and contaminating somatic cells, the semen samples were purified through a discontinuous Percoll (Sigma-Aldrich) gradient (40:80) centrifugation (20 min at 300 g, 25 °C) as described earlier (Kumar et al., 2014). The motile spermatozoa were kept at −80 °C in RNA later (Ambion, Austin, TX, USA) until RNA extraction.

Extraction of total RNA from bovine spermatozoa was carried out using Tri Reagent (Sigma). The protocol was followed according to the manufacturer's recommendation with little modifications. For extraction, the TRI reagent was heated at 60 °C and the samples were incubated for half an hour to completely dissociate the membranes. Equal number of spermatozoa (100 million) was utilized for RNA isolation from each sample. The subsequent steps of the protocol were performed as recommended by the manufacturer. Total RNA sample (aqueous phase) were then passed through a RNA extraction column (GenElute Binding column, Sigma) upon which an RNAse-free, DNAse I treatment (Ambion) was performed in order to eliminate contaminating genomic DNA from the samples. The concentration and the integrity of the total RNA samples were evaluated using Nanodrop spectrophotometer. Total RNA isolated from crossbred bull spermatozoa was reverse transcribed to complementary DNA using Random primers and M-MuLV reverse transcriptase (Protoscript, NEB) according to the manufacturer's instructions. The cDNA product was stored at −20 °C. Genomic DNA contamination was checked by PCR, using intron spanning primer specific to bovine PRM1 gene (Table 1). Bovine genomic DNA isolated from blood by GenElute™ Blood Genomic DNA Kit (Sigma-Aldrich, USA) was used as a positive control. A diluted 1:10 solution of the cDNA was used to elucidate the differential expression of CD9 in mature spermatozoa of good and poor quality crossbred bull. The expression of CD9 mRNA was quantified by real-time PCR (Step One, Applied Biosystems, Foster City, CA, USA). Real-time fluorescence detection method (PCR) was used to quantify the RNA expression of the CD9 gene using CD9 specific intron spanning primers (Table 1). The PPIA (peptidyl prolyl isomerase) and beta-actin gene were used as endogenous controls. The PCR reaction was performed using the SYBR Green Universal PCR Master Mix with ROX, which contains the Taq polymerase enzyme Ampli-TaqGold and all other compounds necessary to carry out the reaction. Following the manufacturer's instruction, 2 μl of cDNA template with concentration of 50 ng/uL, 5 μl 2× SyberGreen Master Mix, 0.5 μl Primer (10 pMol) and 2 μl DNase/RNase free sterile water were mixed in the final reaction volume of 10 μl. All PCR reactions were performed in optical 48 well reaction plates in triplicate for accuracy, and the mean mRNA value was calculated. Negative controls were also run in each set of PCR assays: (1) without cDNA (NTC) and (2) without reverse transcriptase. The absence of PCR products in negative controls was indicative of complete lack of contamination. For PCR, samples were activated at 95 °C for 10 min. Amplification was performed for 40 cycles at 95 °C for 15 s and 55 °C for 60 s. Samples were quantified by the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001). The expression values obtained were normalized against PPIA and beta-actin, as an endogenous 'housekeeping' gene, allowing the comparison of samples independently of the amount of total input cDNA. All determinations were performed in triplicate.

2.5. Analysis of CD9 concentration in bull semen using indirect ELISA

Frozen semen was rapidly thawed at 37 °C, and concentrations of CD9 in seminal plasma were determined by indirect ELISA assay using standard protocol. The total protein level of seminal plasma was measured with BSA protein assay kit. Spermatozoa and seminal plasma were detached by centrifugation at 1000 *g* for 20 min at room temperature. Seminal plasma was further clarified by centrifugation at 10,000 *g* for 20 min at room temperature. Cells were first washed with PBS and lysed with lysis buffer (Sigma Aldrich, USA). At first 96-well plastic microtiter ELISA plates were coated for overnight with coating buffer (pH 9.6) contain antigen. The plates were washed with PBS containing 0.05% Tween-20 (PBST) for three times. Blocking was done with 2% Bovine serum albumin for 1 h at 37 °C. Again washing was done with PBST for three times. One hour incubation was given at

Table 1
Primers designed for the present study.

Gene/marker	Primer sequences	Product size	Annealing temperature
CD9 SSCP	F-5′CCGAAGCAAATTCCACATCATC3′ R-5′TTCACCTGTGGGTGTCTTTC 3′	563 bp	56 °C
CD9 RT	F-5′AAGCTGAAGAACAAGGACGAG 3 ′ R-5′GCAGGTGTCGGTGAGAAAT 3	213 bp	55 °C
PRM1 RT	F-5′AGATACCGATGCTGCCTCAC3′ R-5′GTGGCATGTTCAAGATGTGG3′	234 bp	55 °C
Beta-actin RT	F-5′AGTTCGCCATGGATGATGA 3 ′ R-5′TGCCGGAGCCGTTGT 3 ′	54 bp	55 °C
PPIA-RT	F-5′ATGCTGGCCCCAACACAA 3 ′ R-5′CCCTCTTTCACCTTGCCAAA 3 ′	100 bp	55 °C

37 °C with primary antibody i.e., monoclonal anti-CD9 antibodies produced in mouse (Sigma Aldrich, USA). Then after washing with PBST, plates were incubated for 1 h at 37 °C with conjugated secondary antibody i.e., peroxidase conjugated anti-mouse Fab specific IgG raised in goat (Sigma Aldrich, USA). Substrate o-phenylenediamine dihydrochloride (Sigma Aldrich, USA) diluted in H_2O_2 was added and after development of color, the optical density of the samples was measured at 450 nm within 30 min using a microtiter plate reader (Bio-Tek Instruments Inc., USA).

2.6. Statistical analysis

Genotype frequencies and their combinations were calculated by direct counting. Data pertinent to semen quality parameters (semen volume, sperm concentration and progressive motility) of different genotypic bulls were subjected to ANOVA using the general linear model (GLM) applying SPSS (Statistical Package for Social 89 Sciences) for Window version 11.0.1 SPSS Inc. USA computer software programs according to the following statistical model:

$$Y_{ik} = \mu + G_i + A_k + e_{ik}$$

where, Y_{ik} is phenotypic value of sperm quality traits; μ is the population mean; G_i is fixed effect of genotypes; A_k is fixed effect of age, and e_{ik} is random residual error. Gene expression pattern and concentration of the CD9 gene between good and poor quality semen producers were compared using Student *t*-test.

3. Results and discussion

In the present study, we have associated CD9 gene with certain sperm quality traits including number of sperm/ejaculates, ejaculate volume, spermatozoal concentration and initial progressive motility of semen in crossbreed bulls.

CD9 reported to be expressed in male germ cells with competency of long-term survival as well as cell turnover in the xenogeneic testis (Zohni et al., 2012). As per literature search engines, no reports are available regarding the clinical relevance of CD9 in human male fertility/subfertility. However, one report suggested that CD9 gene variations are not associated with female infertility in humans (Nishiyama et al., 2010).

According to the SSCP results and further sequencing, we have identified three different genotyping patterns at intron 6 region of CD9 gene (Fig. 1). The estimate of the mean of homozygote AA was more than that of estimated for the heterozygote AB in the studied crossbred bull population. No BB genotypes were observed.

Animals carrying AB genotypes were found to have significantly ($P < 0.05$) higher sperm concentration and motility percentage ($P < 0.01$) than AA. However, no significant dominance effect was observed for other selected traits (Table 2). Similarly, polymorphism screening and association of CD9 with male fertility was reported in bulls by Daghigh-Kia (2007), where SNP g.95 T > C in exon 9 showed a positive effect on sperm concentration and sperm motility ($P < 0.05$) in bulls. Here, we reported the SNP at intron 6 region (g.358A > T) of bovine CD9 gene are also associated with sperm concentration. However, Kaewmala et al. (2011) reported that the SNP at intron 6 was significantly associated with sperm motility ($P < 0.001$), plasma droplet rate ($P < 0.001$) and abnormal spermatozoa rate ($P < 0.01$).

Table 2
Association of the different genotypes with bull semen quality parameters (mean ± S.E.).

Genotypes	Number of ejaculates	Volume (ml)	Concentration*	Motility (%)**
AA (n = 50)	112 ± 21	4.5 ± 0.3	891.2 ± 44.2	41.2 ± 3.2
AB (n = 46)	134 ± 24	4.1 ± 0.4	960.9 ± 49.6	52.4 ± 1.1

(*$P < 0.05$ and **$P < 0.01$).

Though the chosen SNP at intron 6 region of CD9 is a silent mutation, it could affect the CD9 function by changing the mRNA stability (Capon et al., 2004). Association of such SNPs at intronic region with certain economical traits may be described by the effect of the intron on mRNA metabolism viz. transcription, editing as well as polyadenylation of the pre-mRNA, translation and degradation of the mRNA product (Le et al., 2003). Furthermore, a number of reports suggested the influence of introns in regulating the expression level of a gene or tissue specific expression pattern (Jiang et al., 2000; Virts and Raschke, 2001; Pagani and Baralle, 2004). However, no significant association could be found for bull semen quality traits except the sperm concentration in the present experiment.

CD9 was reported to be expressed as a murine cell surface biomarker in Leydig cells, Sertoli cells and germ cells within testis, in the epithelial cells of epididymis, vas deferens and prostate gland and in spermatozoa within the lumen of epididymis (Kanatsu-Shinohara et al., 2004). It was also reported that CD9 expressed in the cytoplasm as well as nucleus of spermatogonia, spermatocyte, spermatid and spermatozoa located in the human testis (Jae et al., 2006). Immunochemistry study found that CD9 is distributed in a similar fashion in porcine tissues, being present on testis (Yubero et al., 2010). Very recently, Cupperová et al. (2014) reported that CD9 molecules are not bound to the plasma membrane of spermatozoa but present in the epididymal epithelial secretion. Once, spermatozoa are originated in the seminiferous tubules and additional maturation processes are mandatory before they can participate in fertilization process which occurs in the epididymis gaining the ability to be motile and fertile. No study concerning to differential mRNA expression of CD9 transcript with respect to motility is reported yet. In the present investigation, mRNA expression analysis by qRT-PCR demonstrated that the bovine CD9 gene was differentially expressed among good and poor quality bull semen with respect to motility,

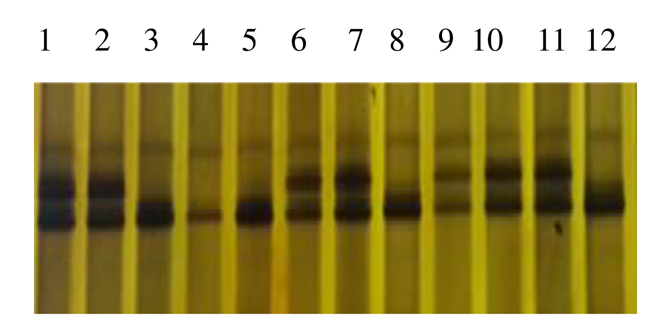

Fig. 1. PCR-SSCP pattern for different genotypes in 15% polyacrylamide gel. Two patterns were obtained and designated as AA, and AB as shown in the figure. Lanes 1, 2, 6, 7, 9, 10 and 11: AB genotypes and Lanes 3, 4, 5, 8 and 12: AA genotypes.

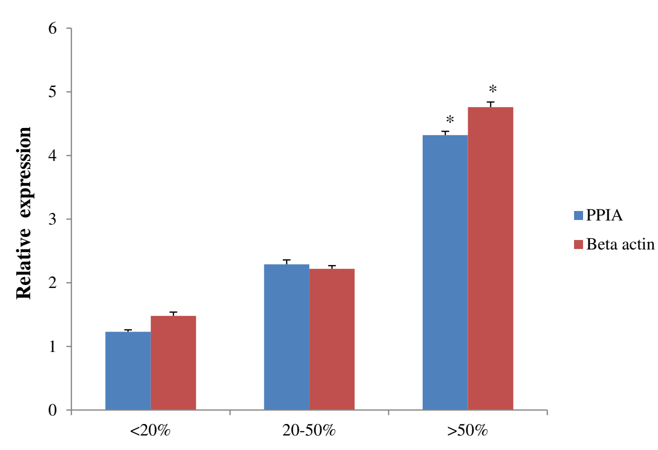

Fig. 2. Relative transcript abundance of CD9 mRNA among motile and impaired bull spermatozoa. PPIA: Peptidyl prolyl isomerase, * indicates significant difference at $P < 0.05$.

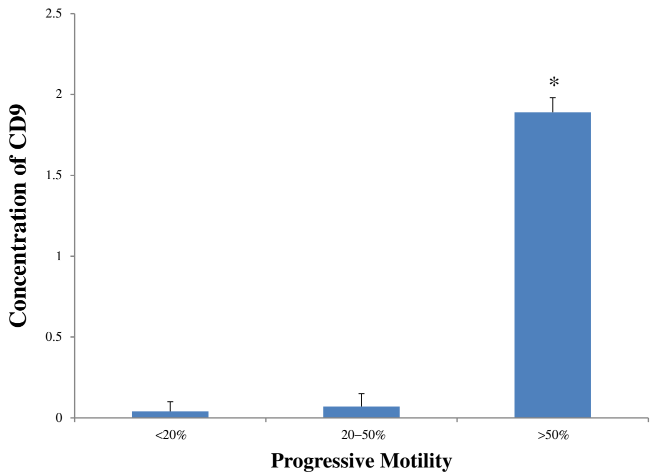

Fig. 3. Concentration of CD9 mRNA among seminal plasma samples exhibiting good and poor sperm motility. * indicates significant difference at P < 0.05.

where the relative transcript abundance were significantly ($P < 0.05$) higher among the crossbred bulls producing good quality semen (progressive motility >50%) than the impaired one (motility <50%) (Fig. 2). Further the concentration of CD9 in good and poor quality bull seminal plasma revealed that progressive motile bull semen have significantly ($P < 0.05$) higher concentration of CD9 (Fig. 3).

However, other SNPs at different exonic/intronic/UTR region of bovine CD9 gene need to be screened for developing its candidacy as biomarker for selecting bulls with better semen quality traits. Further, in the future the study could be extended for large group of animals including the evaluation of CD9 molecule expression in relation to the bull sperm concentration.

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