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RESEARCH NOTE

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Cytosine deletion at AP2-box region of *HSP70* promoter and its influence on semen quality traits in crossbred bulls

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Introduction

Present investigation identified the effect of genetic polymorphism within the AP2 box region 12 located at bovine HSP70 promoter on certain semen quality traits among crossbred bulls revealed the fact that superior capability of wild type promoter genotype over the deletion mutation at HSP70 promoter may partly elucidate better semen quality traits for their contribution in selection of better quality bulls.

Although artificial insemination (AI) is now extensively used in cattle breeding, there is about 40–50% loss in bull semen quality during the freezing–thawing process, including sperm motility, effective survival time and antioxidant enzymes activity (Hu *et al.* 2011). Recently, studies have been carried out in our laboratory to unveil the genetic basis of semen quality traits in cattle (Kumar *et al.* 2014, 2015a, b). It is well known fact that mammalian spermatogenetic cells are sensitive to heat stress (Yin *et al.* 1997). Studies have established that heat shock proteins (HSPs), particularly, the abundantly expressed 70 kDa HSP (*HSP70*), plays an important role in acquired thermotolerance (Nover 1991) and *HSP70* has been suggested to function as an indicator of thermotolerance in cells (Leung *et al.* 1996). *HSP70* is known to play an important role in sperm function after ejaculation and was identified in proteins extracted from sperm of bull (Kamaruddin *et al.* 1996) and boar (Huang *et al.* 1999). Huang *et al.* (1999) reported that both constitutive and inducible forms of *HSP70* are present in ejaculated spermatozoa of boars and both the levels of *HSP70* in spermatozoa and semen quality traits of boars significantly declined during the hot season.

Owing to the ability of *HSP70* to combat heat stress, the study was designed to analyse the genetic variation at AP2 box region of *HSP70* (HSPA1A) promoter and their association with semen quality parameters among Frieswal crossbred bulls (HF × Sahiwal) maintained in India. Our previous studies indicated that mutant variants of AP2 box region of *HSP70* significantly affected cellular thermotolerance, physiological parameters and milk production traits among Frieswal crossbred cows (Deb *et al.* 2013). The present study is the further extension of our earlier reports to investigate the effect of AP2 box variation on the semen quality traits among crossbred bulls.

Materials and methods

A total of 96 mature crossbred bulls maintained at bull rearing unit were included in the present investigation. Semen samples were collected using artificial vagina from each bull during the period, from December 2010 to November 2011. Immediately after collection, the ejaculates were stored at 34°C in a water bath to evaluate the fresh semen quality traits including semen volume per ejaculate (mL), sperm motility (%), viability (%) and sperm concentration (M/mL). The details on the evaluation of good/poor sperm quality can be obtained from our earlier work by Kumar *et al.* (2015c). The fresh semen was then diluted with glycerol–egg yolk–citrate–Tris extender, processed and cryopreserved. After storing 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 postthaw motility (PTM) (%) using light microscopy. Genomic DNA was extracted from the sperm using GenElute™ Blood Genomic DNA kit (Sigma, Aldrich, USA). The DNA samples were

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collected in elution buffer (supplied with kit) and stored at −20°C for future use. Genomic DNA obtained from the ejaculated spermatozoa was subjected to genotyping using a double polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (*Scr*FI) methodology developed earlier by our groups (Deb *et al.* 2013). At base position, in 895 of AP2 box region of *HSP70* (GenBank accession number M98823), deletion of cytosine residue was observed which created a *Scr*FI restriction site. The PCR was performed in a total volume of 100 μL containing ∼0.5 μg genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 250 mM of each dNTP, and 5 U *Taq* polymerase. Initial denaturation was for 5 min (at 94°C) followed by 35 cycles of 94°C denaturation (1 min), 63°C annealing (1 min) and 72°C extension (2 min). The PCR product was detected by electrophoresis in 1% agarose. Two sets of diagnostic primers used for first round amplification to generate *HSP70* gene-specific DNA fragment followed by a mutated set of nested primers (Deb *et al.* 2013) to reamplify the generated PCR products from first round PCR to abolish three native restriction sites for *Scr*FI enzyme and thus, only the diagnostic *Scr*FI site remain intact. Frequencies of the existing polymorphisms in the crossbred bulls were resolute through restriction digestion of the second round PCR products with *Scr*FI restriction enzyme. Different genotypes obtained were subjected for TA cloning using pTZ57R plasmid (InsTAclone PCR Cloning kit, Fermentas, USA). The positive clones were confirmed by restriction enzyme analysis. Positive clone from each genotype was subjected to DNA sequencing. The nested PCR products (encoding the transcription factor binding site) obtained from different genotypic groups were subjected to electromobility assay using Gel Shift Assay kit (Promega, Madison, USA). Briefly, PCR product (1 μL) from each genotype was incubated with HeLA cell extract (9 μL) and subjected to 4% polyacrylamide gel electrophoresis. Silver staining technique was used for visualizing the banding pattern of each group. Appropriate positive (AP2 oligo and HeLA cell extract) and negative (only AP2 oligo) controls were run along with the samples. The effect of the cytosine deletion at AP2 box region of *HSP70* on semen quality parameters, including semen volume, sperm concentration, motility, postthaw motility and viability were investigated using independent *t*-test using SAS 9.3 (SAS Institute, Cary, USA). Specific transformation was applied on semen quality traits, namely, motility, postthaw motility and viability, which was obtained by considering Box and Cox (1964) transformation class and the results were expressed as mean ± standard error in the original scale.

Results and discussion

The double PCR-RFLP using *Scr*FI confirmed the occurrence of homozygous wild-type genotype (CC: 49 and 47 bp) and heterozygous cytosine deletion mutant genotype (C–: 96, 49 and 47 bp) (figure 1, a–c). The genotype frequency of CC was lower (0.47) than C– (0.52) (table 1). The electromobility gel-shift assays of AP2 binding pattern in two different genotypes with nuclear extracts from HeLA cells are depicted in figure 1d. The 96 base pair nested PCR product from each genotype was subjected to gel shift assay and it was concluded that the concentration of free DNA was higher in hetrozygous deletion mutant (C−) than homozygous wild (CC) types. CC genotypes significantly ($P < 0.01$) contribute to better fresh spermatozoal motility (58.78 ± 0.89) and viability (71.83 ± 1.06) as well as postthaw motility (56.87 ± 1.24) compared to C-genotypes (table 1).

Binding of AP2 transcription factor at the Ap2 box region of the *HSP70* promoter sequences were reported by Tacchini *et al.* (1995). Schwerin *et al.* (2003) reported that polymorphisms within the promoter may alter the potential *cis*-acting elements and are also linked with relative thermotolerance in terms of 'productive life time' in Holstein breed of cattle. Further, our earlier studies showed that polymorphism within the promoter region of *HSP70* gene is strongly associated with the thermotolerance and other production traits in crossbred cows (Deb *et al.* 2013). In the present investigation, we have identified polymorphism within the *cis*-acting element of *HSP70* promoter by double PCR-RFLP method among the cross bred bull semen. Our present findings revealed that presence of the heterozygous cytosine deletion mutant genotype (C–) in majority with the complete absence of homozygous cytosine deletion mutant genotypes (−−) in the targeted population. However, Schwerin *et al.* (2003), reported the presence of polymorphism at the same position having all three genotypes in pure Holstein dairy cows in contrast to the presence of only two genotypes in Indian HF crossbred cows (Deb *et al.* 2013). Gel shifting analysis revealed that AP2 transcription factor can interact almost equally at the promoter region of both the genotypes; however, the level of free DNA residues was more in C– genotypes than CC. It may be due to the basis that as the C–DNA are derived from heterozygous, the interaction of transcription factor with promoter is partial. Percentage of normal sperm, motility and sperm concentration are common criteria for evaluating semen quality (Den Daas 1992). Many studies have demonstrated that heat stress during the summer decreases semen quality (Kuo *et al.* 1997). Ejaculated spermatozoa are highly differentiated cells which are short of the biosynthetic machinery to cope with adverse environmental impacts (Hammersdtedt *et al.* 1990). Association analysis with the fresh semen quality traits, the deletion showed significant association ($P < 0.01$) with individual spermatozoal motility and viability traits. Correspondingly, Shrum *et al.* (2010) and Gafer *et al.* (2015) reported that associations between a SNP in *HSP70* promoter and sperm motility as well as velocity traits in bulls, which may be due to persuade of *HSP70* on defense of proteins related to respiration activity and energy level in spermatozoa (Nascimento *et al.* 2008). The study also highlighted that deletion at AP2 box region also significantly ($P < 0.01$) effect on postthaw motility of bull semen after cryopreservation. As the motility of

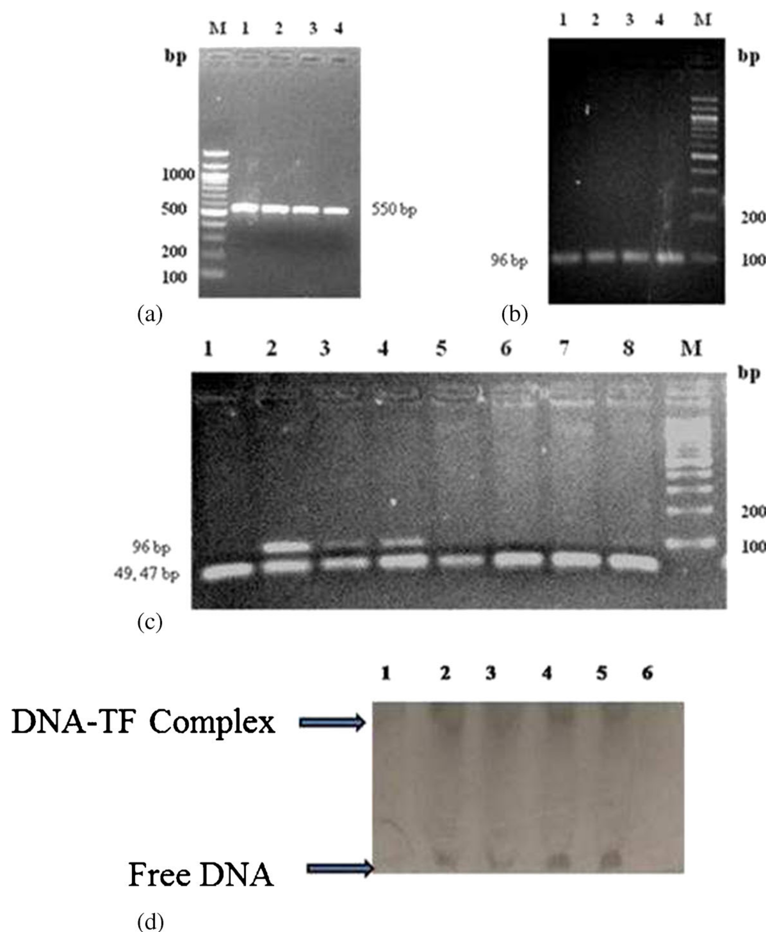

Figure 1. Double PCR-RFLP for genotyping AP2 box region of *HSP70* promoter and its influence on AP2 factor binding. (a) First round (550 bp) PCR with F(W) and R(W) primer and (b) Second round (96 bp) PCR with F(M) and R(M) primer set. M: 100-bp ladder. (c) PCR-RFLP banding pattern of AP2 genotypes. Lane 1, 5–8: CC genotypes (49 and 47 base pair); Lane 2–4: C-genotypes (96, 49 and 47 base pair), M: 100-bp DNA ladder. (d) Electromobility gel-shift assays of AP2 binding pattern in two different genotypes with nuclear extracts from HeLA cells. Lane 1: positive control (HeLA extrac. + AP2 oligo), lanes 2–3: CC genotype (HeLA + PCR product of CC), lanes 4–5: C-genotypes (HeLA + PCR product of C–), lane 6: negative control (only AP2 oligo).

Table 1. Genotype frequency and their association with semen quality traits among crossbred bulls.

		Fresh samples				Cryopreserved samples
Genotypes	Genotype frequency	Volume (mL)	Concentration (M/mL)	Motility (%)**	Viability (%) **	Postthaw motility (%)**
C– ($n = 46$)	0.47	4.36 ± 0.27	970.86 ± 111.11	45.00 ± 2.31	48.64 ± 2.69	33.37 ± 1.87
CC ($n = 50$)	0.52	4.86 ± 0.66	970.07 ± 97.87	58.78 ± 0.89	71.83 ± 1.06	56.87 ± 1.24

**$P < 0.01$.

the postthawed semen indicate its fertility status, we have not studied the viability of postthawed semen samples. Moreover, under Indian climatic conditions, the semen samples are generally evaluated for their viability before freezing and not after freezing. It is a routine practice that the frozen semen samples are evaluated only on the basis of their postthaw motility and not by viability after cryopreservation. However, the study on theviability of the postthawed semen using computer assisted semen analysis (CASA) would add much more knowledge on the fertilizing capacity of the frozen semen samples. Polymorphisms at AP2 box region of *HSP70* gene can influence on semen quality traits and thus, suggest to be used in marker-assisted selection for antiheat stress as well as superior reproductive bulls. Nevertheless, further study on the evaluation of viability of the postthaw semen samples using CASA needs to be conducted to support the results obtained in the present study. Further, the present results could be validated even in larger bull populations using CASA to find the effect of deletion at AP2 box region on postthaw motility and viability parameters of cryopreserved semen before utilizing it as an early marker for selection of bulls in artificial insemination programmes.

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