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Short Communication

Nucleotide Variationin Cation Channel of Sperm 1 Gene in Vrindavani Cattle

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ABSTRACT

The present study was carried out on Vrindavani cattle, to investigatethe nucleotide variations in exon 3 and 4 of Cation channel of Sperm 1 (CatSperl) gene by the PCR–RFLP technique and the nucleotide sequencing of respective amplicons. To detect restriction site polymorphism, PCR–RFLP was performedon genomic DNA isolated from blood of 100 randomly selected crossbred animals. The PCR–RFLP of 482 bp encompassing complete exon 3 and 4 with flanking introns, using *Pvu*II and *Rsa*Irestriction enzymes produced two (403 and 79 bp) and three bands (229, 152 and 103 bp), respectively. This fragment of CarSperl gene exhibited absence of polymorphism with respect to restriction enzymes used and accordingly, the allelic frequency was found to be 1.0. The monomorphic pattern the amplicon of CatSperl gene with respect to different enzymes indicated the conservednessof this gene. Ampliconswere subjected to DNA sequencing after which it was annotated and submitted to GenBank. In order to study the variations at nucleotide level, the sequences of studied amplicons were compared with similar sequences of Zebu cattle, buffalo and goat.

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Indiahas been witnessing steady increase in crossbred cattle population,which is contributing significantly to the success of dairy industry. To sustain the increasing population of crossbreds, more doses of crossbred semen are required. Semencryo–preservation becomes a tool of importance in such a situation.The ultimate goal of semen preservation has been to obtain pregnancies after artificial insemination, which would be as effective as natural mating. The successful preservation of semen depends on several factor, which are involved in the process of cryopreservation and the term 'freezability' has been given to mean the ability of semen to survive after being frozen without suffering substantial damage. The damage to the semen expressed as sperm count, motilityand fertility but practically, it largely refers to post thaw motility (PTM) (Ravimuruganet *al*., 2007) which again depends on initial/pre–freeze motility and is the ultimate trait which qualifies the semen for artificial breeding. Crossbred bulls have high percentage of abnormal spermatozoa, lower level of sperm motility and viability, together causing decline in fertility rate (Dhanjuet *al*., 2006). Poor sperm motility and freezability of semen has also been reported in Vrindavani crossbred bulls (Ghosh *et al*., 2007). Researchers have reported more than 50% rejection rate in different crossbred bulls (Chacon *et al*., 1999; Tyagiet *al*., 2006) due to poor quality,especially the sperm motility. Many genes are known to control sperm motility.Off late, an ion channel gene was identified which had a significant bearing on sperm motility (Ren *et al*., 2001; Quill *et al*., 2001). Thiswas named as Cation channel of sperm (CatSper) and reported to have four subunits.CatSper family mutations resulted in male infertility (Darszonet *al*., 2006).CatSpers1–4 are expressed in testis and localized primarily to the principal piece of sperm tail (Ren *et al*., 2001; Quill *et al*., 2001; Lobleyet *al*., 2003; Jin *et al*., 2005). CatSperl and 2 are required for the hyperactivation of sperm cell motility, which is essential for fertility(Qi *et al*., 2007). This study was therefore taken up, to ascertain nucleotide variation inCatSperl gene inVrindavani crossbred cattle, so as to initiate a step in searching the promising DNA marker that could be developed to improve sperm motility of crossbred cattle by assisting in bull selection process.

A total of hundred and five randomly selected Vrindavani cattle(50–62.5 % exotic inheritance comprising of Holstien Frisian, Brown Swiss, Jersey; with Hariana as indigenous stock) developed at the Indian Veterinary Research Institute, Izatnagarwere included in the present investigation. A total of 15 ml of venous bloodwas collected from each crossbred animal in sterile 15 ml polypropylene centrifuge tube containing 0.5 ml of 2.7% EDTA as anticoagulant. DNA isolation was performedby phenol chloroform extraction method (Sambrook and Russel, 2001) and the precipitated DNA was dissolve in 200µl of TE buffer. The quality and concentration of genomic DNA was evaluated by spectrophotometer (PG Instruments, UK). Samples that showed an OD ratio (OD_{260}/OD_{280}) in the range of 1.7 to 1.9 were assessed to be of good quality.

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Primerswere designed by the help of DNASTAR software to amplify CatSper 1 gene on the basis of already reportedsequence (*in silico* generated/ predicted)in *Bostaurus* cattle (NC_007330) available in the GenBank (www.ncbi.nlm.nih.gov).The fragment of CatSper 1 gene (482bp) comprising of complete exon 3 and 4 with flanking introns, was amplified by using a pair of self designedprimers (Forward: 5' CCT CAC CAC GGC GAA CAC CAC 3' and Reverse: 5' GGA CTA CAC CAG CAG GGG AGA GC 3'). The stock solution was prepared by diluting the same with DNase free water (Biogene, USA) in such a way that each has concentration of 300pmoles/µl. This was kept at 4°C for 2–3 days for allowing complete dissolution of primers. The working primer solution was further prepared by 10 fold dilution of stock primer solution in DNAse free water (Biogene, USA) so that each has a concentration of 30 pmoles/µl.A master mix was prepared for the required number of reactions by adding the reaction components in the following order, autoclaved distilled water, 10X assay buffer, dNTPs, $MgCl_2$,forward and reverse primers,and finally *Taq* DNA polymerase enzyme. The 24µl of master mix was distributed into 0.2 ml of each PCR tubes duly labeled and marked. Finally, 1 µl of genomic DNA was added to the PCR tubes followed by gently mixing. The PCR tubes with the reaction mixture were put in a thermocycler (iCycler, Biorad, USA). The reaction mixture and PCR programme were followed as suggested by Sharma *et al.* (2009, 2010), to achieve the satisfactory level of amplification in a final volume of 25µl containing genomic DNA (60–100ng), 2.5µl of 10xPCR (1.5mM), 2.5 µl of dNTPs mix (0.2mM), 1.5 µl of $MgCl_2$ (1.5mM), 1µl each forward and reverse primers and 0.2µl of Taq DNA polymerase (5 U/µl).Samples were amplified for 35 cycles with initial denaturation at 94 °C for 3 min., cyclic denaturation 94°C for 1min., annealing at 62°C for 1min., cyclic extension 72°C for 1min., final extension 72°C for 10min.

Theamplicon was digested by *Pvu*I and *Rsa*Ienzymes, which had the recognition frames as CAG↓CTG and GT↓AC respectively. The digested products were electrophoresed in 2.5% w/v agarose gel, stained with ethidium bromide, at 100 V for 5min and then 90V for 1 hr in 1x TBE buffer and visualized under UV light. The amplicon of CatSper1 gene was eluted, cloned in pGEMT vector and sequenced in both orientations. The obtained sequence on crossbred cattle was then aligned with similar sequences in other species viz. zebu cattle (*Bosindicus*), buffalo (*Bubalusbubalis*) and goat(*Capra hircus*) using MEGALIGN module of DNASTAR software (Lasergene, USA).

The digestion of 482 bpamplicon of encompassing exon 3 and 4, with *Pvu*II produced two bands of 403 and 79bp (Figure 1). On digestion of same PCR products with*Rsa*I gave three bands of 229, 152 and 103bp (Figure 2) respectively. This restriction pattern was due to the presence of a single*Pvu*II site (CAG↓CTG) at the 403rd position and two *Rsa*I site (GT↓AC) at the 103rd and 255th position of amplicon. The DNA samples showed the same restriction pattern depicting the presence of same RE site in all animals. The results suggested that this amplicon was monomorphic, since all of them showed a single homozygous genotype.Monomorphic pattern has also been observed in exon 2 using *Eco*RI, *Hind*IIIand exon 5 using*Alu*I and *Taq*I restriction enzymes of CatSper1 gene in Vrindavani cattle (Geetha*et al.*, 2011, 2013). However, polymorphism was observed in this gene by using PCR–SSCP method by Modi*et. al.* (2011, 2014).Sivakumar*et al.* (2013a, 2013b) also observed various SNPs in CatSper1 and CatSper2 genes in vrindavani as well as tharparkar cattle.

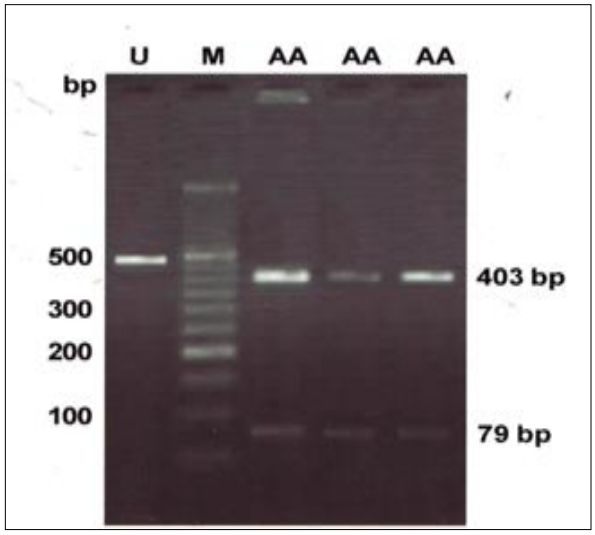

Figure 1: CatSper1 RFLP–482bp amplicon digestion with *Pvu*II; U: Undigested; M: 50 bp marker

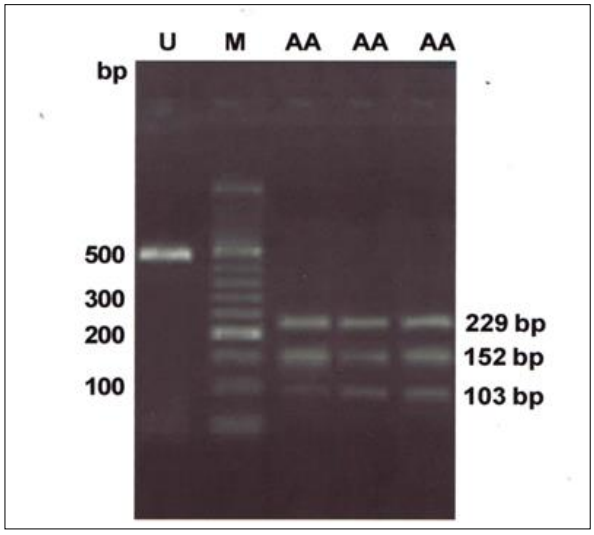

Figure 2: CatSper1 RFLP–482bp amplicon digestion with *Rsa*I; U: Undigested; M: 50bp marker

Though the 482 bpamplicon encompassing exon 2 and 3 along with intermediary and flanking introns of CatSper1 gene in crossbred (vrindavani) cattle, zebu (tharparkar), buffalo (murrah), goat (black bengal) weresuccessfully amplified however the sheep (*Ovisaries*) and mithun (*Bosfrontalis*) DNA samples could not be amplified. The

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sequences obtained from these amplicon from these four species (GenBank accession no. JF737759–62) were compared. No variations were noticed between crossbred and zebu cattle. However three nucleotide variations have been noticed in crossbred cattle when compared to buffalo and all were found in intron 3 (Figure 3a, 3b). On comparison of crossbred cattle with goat, three nucleotide variations were observed in the exon 3 and two in intron 3, six in exon 4 and three variations were noticed in intron 4 region. One deletion was witnessed in goat at 374thnucleotide position (Figure 3b)as compared to other ruminants which had a C nucleotide at that position. Three amino acid variations have been noticed in goat. The nucleotide and amino acid sequence homology of crossbred cattle was found to be highest (100%) with zebu cattle (Figure 4).

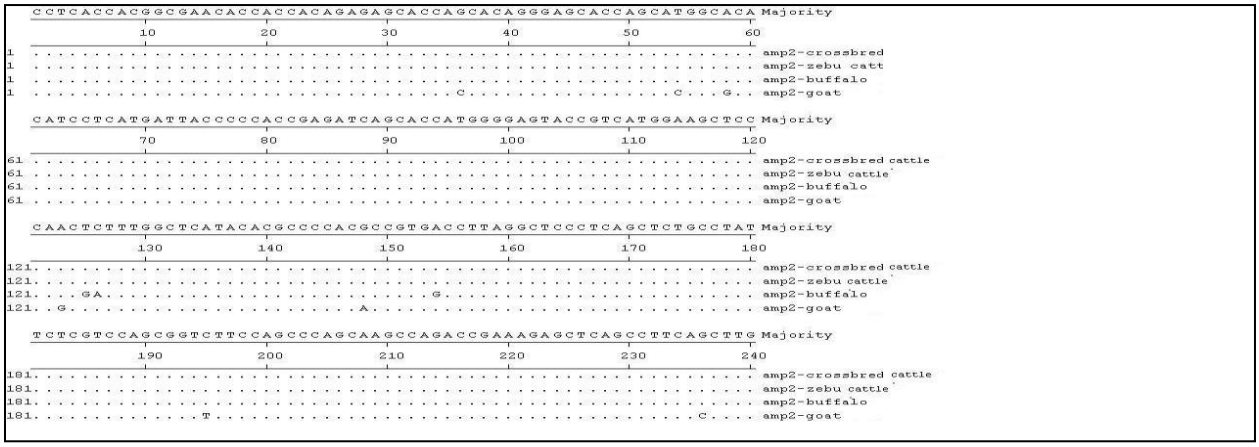

Figure 3(a): Comparative analysis of nucleotide sequence of 482bp fragment of CatSper1 gene

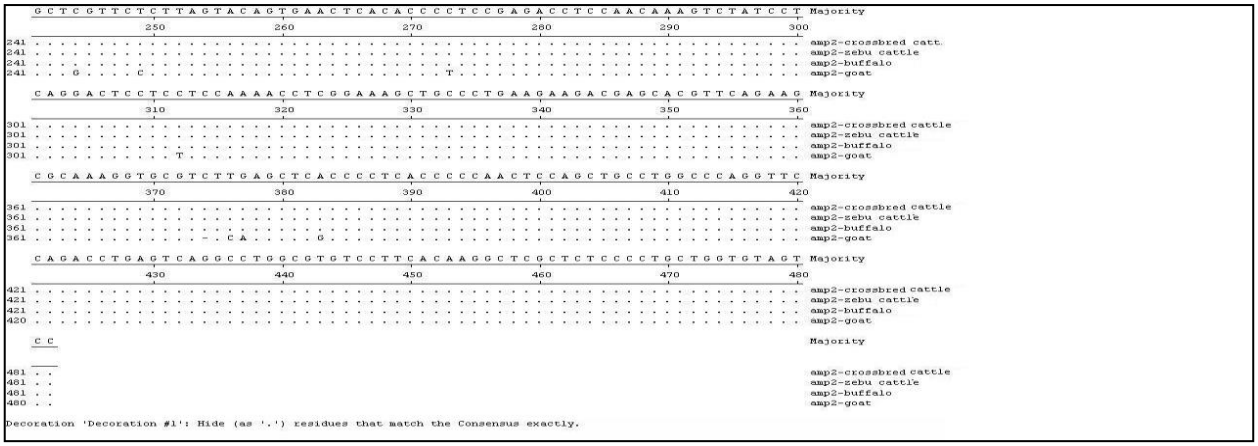

Figure 3(b): Comparative analysis of nucleotide sequence

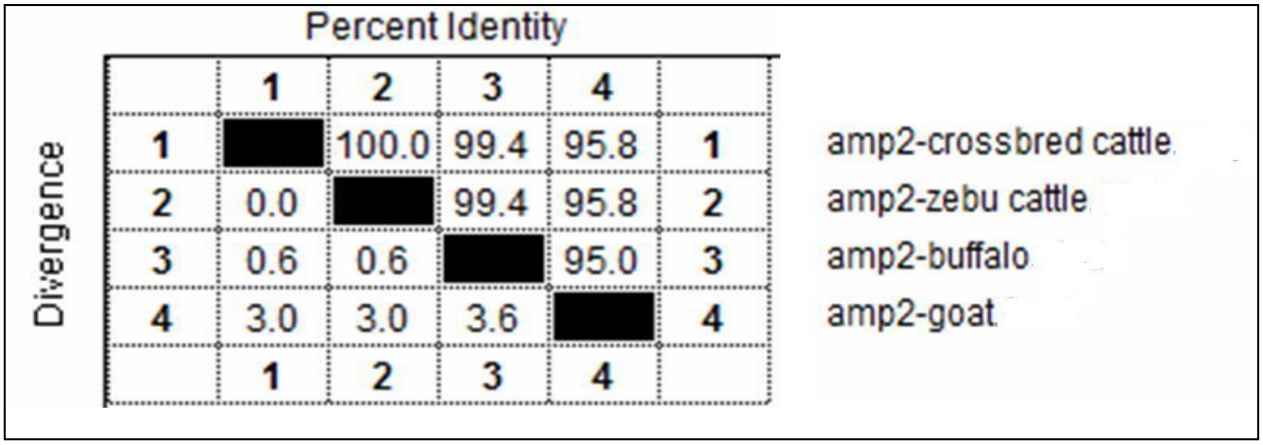

Figure 4.Homology of Nucleotide Sequence of 482bp fragment of CatSper1 Gene

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Poor semen quality is the main cause of high rejection rate in crossbred bulls (Mathew *et al*., 1982; Rao and Rao, 1991 and Kumar, 2006). Methods have been developed and demonstrated to improve and measure the potentiality of sperm function however importance of candidate gene marker cannot be obviated (Kumar *et al*. 2013a, 2013b).Genetic characterization and polymorphism identification of CatSper1 is prerequisite for finding a genetic marker of this gene, which may help in improvement of sperm motility and freezability in crossbred cattle. The monomorphic pattern of exon 3 and 4 of CatSper1 gene with respect to different enzymes indicated the conservednessin these exons and suggested to explore polymorphism in other coding regions of this gene as well as other genes responsible for sperm motility. Identification of alleles that are in high association with poor sperm motility in crossbred bulls is the most important area of research now a days and search of a genetic marker in this regard is much needed.

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