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Nucleotide variability of protamine genes influencing bull sperm motility variables

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ABSTRACT

Protamines (PRMs), important proteins of chromatin condensation in spermiogenesis, are promising candidate genes to explore markers of sperm motility. The coding and *in-silico* predicted promoter regions of these genes were investigated in 102 crossbred and 32 purebred cattle. Also, mRNA quantification was done to explore its possibility as diagnostic tool of infertility. The PCR-SSCP analysis indicated there were two band patterns only in fragment I of the *PRM1* and fragment II of the *PRM2* gene. The sequence analysis revealed A152G and G179A transitions in the *PRM1* gene. Similarly, G35A, A49G and A64G transitions were identified in the *PRM2* gene which resulted in altered amino acid sequences from arginine (R) to glutamine (Q), from arginine (R) to glycine (G) and from arginine (R) to glycine (G), respectively. This caused the reduction in molecular weight of *PRM2* from 2157.66 to 1931.33 Da due to reduction in the number of basic amino acids. These altered properties of the *PRM2* protein led to the reduction in Mass Motility (MM: $P < 0.01$), Initial Progressive Motility (IPM; $P < 0.05$) and Post Thaw Motility (PTM; $P < 0.05$) in crossbred bulls. The least squares analysis of variance indicated there was an effect of *PRM2* haplotypes on MM ($P = 0.0069$), IPM ($P = 0.0306$) and PTM ($P = 0.0500$) in crossbred cattle and on PTM ($P = 0.0408$) in the overall cattle population. Based on the RT-qPCR analysis, however, there was not any significant variation of *PRM1* and *PRM2* gene expression among sperm of Vrindavani bulls with relatively lesser and greater sperm motility.

1. Introduction

During mammalian spermiogenesis, one of the visible changes that occurs is substantial condensation of the chromatin structure (Aoki et al., 2006c). During this process, transition proteins replace histones which in turn are replaced by protamines in elongating sperm (Cynthia et al., 2004). These spermiogenesis events are supported by specific proteins such as sperm nuclear proteins including protamines (PRMs; Tanaka and Baba, 2005). Protamines have an important role in condensation of chromatin and complete inactivation of transcription (Aoki et al., 2006d; Luke et al., 2014). The genes for protamine 1 (*PRM1*) and the family of protamine 2 (*PRM2*) proteins (*PRM2*, *PRM3* and *PRM4*) are expressed in sperm cells (Aoki et al., 2006a,b; Depa-Martynow et al., 2007a,b). The

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PRM1 and *PRM2* are the abundant core proteins contained in the sperm head. These proteins are important in packaging or protecting the male genome from damage during and after the histone – protamine transition (Corzett et al., 2002; Cho et al., 2003; Lewis et al., 2003; Nasr-Esfahani et al., 2004; Aoki et al., 2005; Laberge and Boissonneault, 2005; Kempisty et al., 2006; Carrell et al., 2007; Kempisty et al., 2007; Kotwicka et al., 2007). The normal *PRM1* and *PRM2* ratio was associated with a greater motile sperm count, greater percentage of progressively motile sperm, and greater fertilisation capacity (Rogenhofer et al., 2013). An abnormal structure of protamines led to infertility or poor reproductive outcomes (Clark and Civetta, 2000; Carrell and Liu, 2001; Cho et al., 2001; Agarwal and Said, 2003; Tanaka et al., 2003; Aoki et al., 2005; Miyagawa et al., 2005; Iguchi et al., 2006; Carrell et al., 2007; Ravel et al., 2007; Carrell, 2008; Gazouez et al., 2008; de Mateo et al., 2009; Grassetti et al., 2012; Zini and Libman, 2006) through decreased sperm number, motility and viability (Aoki et al., 2006d), increased chromatin damage (Aoki et al., 2006d; Mengual et al., 2003), and embryo mortality (Cho et al., 2003). The assessment of PRM gene expression has been conducted in crossbred and purebred bulls (Ganguly et al., 2013; Parthipan et al., 2017) which revealed non-significant differential expression of PRM genes. Also the PRM2 gene was marginally transcribed and translated in bull and boar spermatids (Maier et al., 1990). Protamines, therefore, are genes that could be assessed for identifying markers of relatively greater values for sperm motility variables in cattle.

Cattle, especially crossbreds, have an important role in increased milk production in India. Crossbreds, however, have a greater disease susceptibility, increased reproductive problems, repeat breeding, and inferior semen quality owing to poor freezing capacity, cryo-injuries, poor motility and viability, greater percentage of dead and abnormal sperm, and initial/pre-freeze motility (Venkatasubramanian et al., 2003; Dhanju et al., 2006; Martin et al., 2012) which result in a reduction in reproductive efficiency (Rao and Rao, 1991; Kumar, 2006; Ghosh et al., 2007). Also, the number of proven *Bos indicus* bulls available is modest. It is, therefore, impossible to meet present and prospective demand for semen unless a rigorous approach is taken for identifying superior bulls. An alternate strategy would be to select genetically elite bulls, in which the importance of candidate gene markers cannot be ascertained. Hence, this study was conducted to explore nucleotide variabilities in *PRM1* and *PRM2* genes and to profile the expression in bull sperm along with the effect on sperm motility variables in bulls.

2. Materials and methods

2.1. Genetic stocks, semen collection and isolation of DNA

Prior approval of institute's animal ethics committee was obtained for all procedures of the experiment. Random selection occurred of 139 bulls [107 crossbred (*Bos taurus* x *Bos indicuss*) and 32 purebred (*Bos taurus* and *Bos indicus*)] maintained at the Germ Plasm Centre (GPC) of Indian Veterinary Research Institute (IVRI), Izatnagar; Animal Breeding and Research Center of National Dairy Research Institute (NDRI), Karnal; Animal Breeding Center, Salon; Deep Freezing Station, Gajaria, Lucknow; Deep Freezing Station, Lakhimpur and Sabarmati Ashram Gaushala, Bidaj were included in this investigation. About 2 ml of fresh semen samples of Vrindavani bulls were collected in sterile microcentrifuge tubes at the Germ Plasm Center of IVRI. There were 10–15 straws of frozen semen samples were procured from different semen stations of India. These semen straws were collected and transported to IVRI, Izatnagar in a liquid nitrogen container (Cryocan) and stored until the isolation of DNA. Genomic DNA (gDNA) was isolated from the fresh as well as frozen semen samples using phenol-chloroform extraction method (Sambrook and Russel, 2001). These isolated gDNA samples were subjected to routine quality, quantity and purity assessment by 0.75% agarose gel electrophoresis and NanoDrop spectrophotometer (NanoDrop1000, Thermo Scientific, USA). The gDNA samples of good quality, purity and concentration were used for further analysis. The final concentration of genomic DNA was adjusted to a concentration of 100 ng/μl.

The profiling of *PRM1* and *PRM2* gene expression was conducted on Vrindavani bulls (*Bos taurus* x *Bos indicus*) which were developed by the cross of Holstein Friesian, Brown Swiss, Jersey (taurine breeds) with Hariana (indicine breed) at the IVRI, Izatnagar, India with an amount of exotic inheritance varying between 50% and 62.5%. Approximately 2 ml fresh semen samples were collected from selected Vrindavani crossbred bulls maintained at the Germ Plasm Centre (GPC) of IVRI in a DEPC treated sterile microcentrifuge tubes for RNA isolation. The samples were transported to laboratory at 4 °C in an icebox containing ice packs and were processed immediately.

2.2. In silico prediction of promoter region of PRM genes

Binding of transcription factors to promoter region is an important component in expression of genes in response to any stimuli and promoter region is the primary regulatory region for gene expression. Hence, identification of elements of the promoter region is an important component in understanding the mechanism of gene expression regulation. The Neural Network Promoter Prediction program of the Berkeley Drosophila Genome Project (BDGP) was used in this study to predict promoter (Reese, 2001). Only the 500 bp upstream region from the start codon of the published sequence of *Bos taurus* was used to predict the promoter region and transcription start site in *PRM1* and *PRM2* genes. For selection of predicted promoter sequences, a cut off score of 0.8 was used and the promoter sequences above this cut off value were considered. The consideration of 500 bp or less of the upstream region from the start codon was used due to the restriction of fragment size for Single Strand Conformation Polymorphism (SSCP) analysis. To amplify these predicted promoter sequences of *PRM* genes, one set of primers was designed.

2.3. Primer designing and amplification of PRM genes

Promoter and coding regions of *PRM1* and *PRM2* genes were amplified in the gDNA samples of crossbred and purebred cattle