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Genetic polymorphism of FSHβ and PPP1r11 gene in crossbred breeding bulls

S J SAYYED[1], M M CHOPADE[2✉], S SAJID ALI[3], P V NANDEDKAR[4], P M KEKAN[5]
and M D KHARAWADKAR[6]

College of Veterinary and Animal Sciences, Parbhani, Maharashtra Animal and Fishery Sciences University, Nagpur (Maharashtra)

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Livestock sector is the backbone of agriculture in India, which contributes to about 8.8 per cent of employment in rural population and supports two-thirds of rural households. It contributes significantly to the Indian agricultural economy and provides a substantial portion of agricultural output in terms of value, with dairy farming being a major contributor. Therefore an appropriate breeding program is the crucial requisite for development of livestock sector. Any breeding program's ability to produce quality offspring depends on how many superior individuals are there in the flock, which in turn depends on individual fertility. The production of elite animals in livestock breeding is essential for driving genetic improvement, increasing productivity and profitability, enhancing disease resistance and conserving genetic diversity. Elite animals belonging to indigenous breed possess unique genetic traits and adaptations. Selective breeding of these animals helps to conserve genetic diversity within the livestock population and preserve valuable traits for future generations. Fertility, in this regard, is an important reproductive trait which contributes to the propagation of elite germplasm. In livestock breeding programs, fertility is a critical factor in selecting breeding stock. High fertility rates are desirable traits that breeders aim to perpetuate in subsequent generations to enhance productivity and profitability. Both male and female fertility are integral components of successful breeding strategies aimed at optimizing productivity, genetic improvement and sustainability in livestock production. However, male fertility is often emphasized due to its greater influence on production system. Bull fertility is the cornerstone of a successful breeding program as the male contributes about half of the progenies to the population. The FSHβ and PPP1R11 are some of the important genes responsible for male fertility. Follicle-stimulating hormone (FSH) is a glycoprotein that is synthesized in the pituitary and controls mammalian reproduction. FSH and testosterone both work together to control sertoli cell function, which is essential for the production and maintenance of both quality and quantity of sperm during spermatogenesis in males. The spermatogenesis is regulated by (FSH) acting on germinal cells in the seminiferous tubules of the testis up to the secondary spermatocyte stage, Androgens derived from the testis stimulate the later stages of spermatogenesis. The gene PPP1R11 (Protein Phosphatase 1 Regulatory Inhibitor Subunit 11) regulates the activity of protein phosphatase-1. Its diverse functions in spermatogenesis are suggested by varied isoforms as well as cellular sites. It plays an important role in sperm motility and spermatogenesis. Therefore, considering the importance of these genes in bull fertility, the present investigation has been carried out to screen these genes for fertility in crossbred breeding bulls.

Blood samples from sixty crossbred breeding bulls reared at Frozen Semen Lab (FSL) Pune, Aurangabad and Nagpur divisions of Maharashtra region were collected for the present study. Approximately, 3 ml of whole blood was collected aseptically from the jugular vein of breeding bulls for DNA isolation in sterile blood vacutainers containing EDTA as an anticoagulant and transported to the laboratory by properly maintaining the cold chain. DNA was isolated from the blood samples by using the standard Phenol: Chloroform: Isoamyl alcohol method described by Sambrook and Russell (2001). The quantity and quality of genomic DNA were checked using a nanodrop spectrophotometer and by running the DNA on agarose gel electrophoresis, respectively. The blood samples with a value of OD260/280 ranging between 1.70 to 1.90 were considered for further investigation. Stock DNA was diluted in Triss EDTA (10 mM Tris-HCL and

Present address: [1]M V Sc Scholar, Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Parbhani. [2,3,4]Associate Professor, Department of Animal Genetics and Breeding, College of Veterinary and Animal Sciences, Parbhani. [5]Associate Professor and Head, Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Parbhani. [6]Associate Professor, Department of Livestock Production and Management, College of Veterinary and Animal Sciences, Parbhani. ✉Corresponding author email : drmmchopade@gmail.com, Mobile No - 9421117835/9136391864

1 mM EDTA) buffer to make a working concentration of about 50ng/μl. The integrity of the isolated genomic DNA samples was checked by running these DNA samples on 0.8% agarose gel electrophoresis at 90 volts staining with ethidium bromide dye for visualization of the DNA bands under UV trans-illuminator. From these isolated DNA samples with minimal smearing and suitable for further usage were selected.

It was observed that optical density ratio for all isolated samples ranged from 1.66 to 1.89, with the mean of 1.80 ± 0.01 indicating high purity of the extracted DNA. Divya (2016) had earlier reported purity of DNA ranged between 1.60 to 2.19 with a mean of 1.90 ± 0.01. Similarly, Raina (2016) and Sena (2021) obtained the samples with OD range of 1.7- 1.9. The DNA concentration was ranged from 25 ng/μl to 349.5 ng/μl. The yield and specificity of the DNA samples did not significantly differ between samples based on visual inspection in an agarose gel. The low concentration samples were adjusted by increasing DNA quantity while higher DNA concentration samples were diluted by adding proportionate amount of nuclease-free water. Samples with very low concentrations were omitted from the study and a standard concentration of 50 ng/μl was used for further analysis. Sena (2021) reported DNA concentrations ranging between approximately 90-340 ng/μl. Kadam *et al.* (2020) also obtained the DNA concentration in the similar range as of present study. However, Raina, (2016) obtained DNA concentration ranging between 510-3004 ng/μl, which was comparatively very high compared to the DNA concentration used in this study.

The working solution of primers was prepared and optimized at 57°C and concentrations were kept as it is to run the gradient PCR. The forward primer used for amplification of FSHβ gene was 5`CTTCCAGACTACTGTAACTCATC3' while reverse primer was 5`GTAGGCAGTCAAAGCATCCG3'. For the PPP1R11 gene, the forward and reverse primers used were 5`AGCGCTTTGACGCATTTAGT3' and 5`GCCAAGTTCCCAGTCTTTCA3', respectively. The PCR protocol used for amplification of FSHβ gene was initial denaturation at 95°C for 5 min, denaturation at 95°C for 0.30 min, annealing at 63°C for 0.30 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. Similarly, for PPP1R11 gene, initial denaturation at 94°C for 5 min, denaturation at 94°C for 1 min, annealing at 59.4^{o}C for 1 min, extension at 72°C for 0.45 min and final extension at 72°C for 5 min. The 25 μl PCR mix for both these genes was prepared by using template DNA, forward primer and reverse primer 1.0 μl each, PCR master mix (Taq DNA polymerase) 12.5 μl and Nuclease free water 9.5 μl. PCR cycles were repeated for 35 times. The PCR products (10μl) from each tube were digested with 5 units of restriction enzymes. The genotypic frequencies and gene frequencies were estimated and sequencing was carried out for confirmation of PPP1R11 cutting site.

The concentrations of the forward and reverse primers were adjusted by performing PCR experiments. It was observed that when primers were used at a lower level than optimal, desired product was not produced. This could be because there may not be proper binding of primer with DNA due to insufficient primers available. (Sambrook and Russell, 2001). If the primer concentration used was high, smearing was observed in PCR product, so the standardized amount (10 pmol/μl) was used. Dai *et al.* (2009), Jain (2016), Shabahat *et al.* (2016), Paul (2016), Patil (2016) and Sena (2021) also used 10 pmol/μl primer's concentration. However, in contrast, Raina *et al.* (2016) and Dalvi *et al.* (2018) used 20 pmol/μl primer's concentration for amplification of FSH- β gene.

The DNA fragment of 313 bp for FSH-β gene and 599 bp for PPP1R11 gene was successfully amplified (Figure 1 and Figure 3) and subjected to restriction digestion. The enzyme *Pst1* and *NcoI* were used for digestion of FSH-β and PPP1R11 genes, respectively. The *Pst1* enzyme reported the cutting site at position 5'…CTGCA^G…3' in FSH-β gene. Ishak *et al.* (2011), Budi (2018) and Sena (2021) also reported the presence of a similar cutting site for the FSH-β gene. The sample after running on 2.7 % agarose gel electrophoresis revealed homozygous AA (202bp & 99bp), heterozygous AB (313bp, 202bp, 99bp) & homozygous BB (313bp) genotypes as shown in Figure 2 with the genotypic frequencies of 0.05, 0.16 and 0.78, respectively. The allelic frequencies of allele A was 0.13 while allele B was 0.87. The results of FSH-β gene analysis showed that B allele and BB genotype was predominantly observed in crossbred bulls.

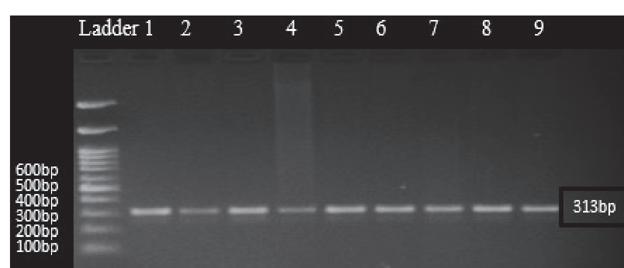

Fig. 1. PCR amplified DNA product of FSHβ gene

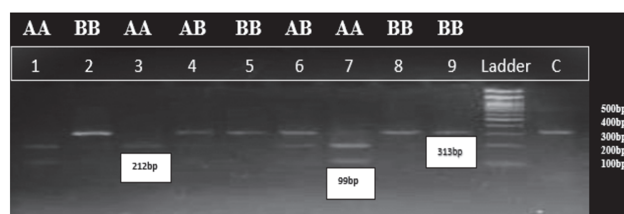

Fig. 2. PCR – RFLP of FSH-β gene showing three genotypes

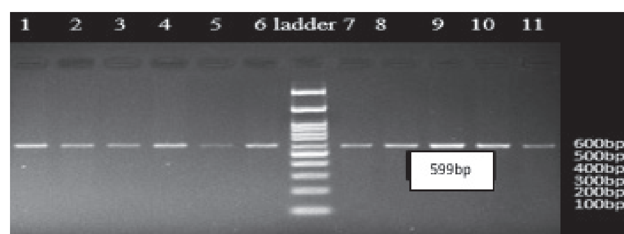

Fig. 3. PCR amplified DNA fragment of PPP1R11 gene

The variations were reported at allelic level A and B as well as at genotypic level AA, AB and BB. The highest frequency of allele B (1.0) and genotype BB (1.0) was reported by Ishak *et al.* (2011) in Bali cattle while the highest frequency of allele A (0.65), genotype AA (0.38) and AB (0.54) was reported by Dalvi *et al.* (2018) in crossbred cattle. Kadam *et al*. (2015) reported two genotypes with genotypic frequencies of BB and AB genotypes as 0.404 and 0.595, while the gene frequency for A and B allele was 0.702 and 0.307 in crossbred cattle. Similarly, Dalvi *et al.* (2018) estimated genotypic frequencies 0.38, 0.54 and 0.08 for AA, AB and BB genotypes while gene frequency of A allele was 0.65 and B allele was 0.35 in crossbred cattle. The association studies of the polymorphism with semen quality and quantity traits were not carried out in the present study for FSH-β gene as the data could not be procured from FSLs due to administrative difficulties, however the literature referred indicates that AA and BB genotypes were associated with abaxial and macrocephalus type of sperm abnormalities while genotype AB was associated with only abaxial type of sperm abnormalities (Ishak *et al.* 2011). It was also reported that the sperm abnormalities have been associated with male infertility and sterility in most of the species studied and the abnormalities of morphological defects that are evident upon clinical examinations. The sperm structure in general plays a substantial role in fertilization as well as pregnancy outcome (Chenoweth, 2005). Although fertility in males is a character with low heritability, certain aspects of bull fertility, including sperm morphological abnormalities, are under the control of genes. Chenoweth (2005) and Freneau *et al.* (2010) divided the sperm abnormalities into major and minor types, those caused by genetic factors include mainly abnormalities due to tail, round head and knobbed acrosome defect, which occur in the acrosome region of sperm.

Another DNA fragment of 599 bp for PPP1R11 gene was digested with *Nco*I enzyme. The presence of a restriction site using the *Nco*I Restriction enzyme at position 267 in all samples resulted in the 599 bp fragment being cut into two fragments of 332 bp and 267 bp, revealing one restriction site for *Nco*I (Figure 4). Paul (2016) used the same restriction enzyme in 87 Sahiwal bulls but did not find the restriction cutting sites, while Jain (2016) also used PCR-RFLP in Murrah bulls with a restriction enzyme *EcoNI* and

Fig. 4. PCR-RFLP of PPP1R11 gene showing two DNA fragments

Fig. 5. Clustal-W alignment showing mutations at position 186 (T>G), PPP1R11 gene.

Fig. 6. Chromatogram sequencing showing mutations at position 186 (T>G)

reported uncut single band of DNA fragment. Hence, in the present study, the cutting site was confirmed with the help of sequencing of representative samples. The sequencing results also revealed the transversion mutation in PPP1R11 gene where the allele ‘T’ was replaced by allele ‘G’ at the position 186 (Fig. 5 and Fig. 6). Shabahat *et al*. (2016) reported one SNP T28708860C mutation in Karan Fries bulls. Jain (2016) also reported variation at 56 position in PPP1R11 gene in comparison to the reference sequence of Bos taurus. Out of which, 32 were transition type and 24 were transversion type. Two variations were found in 5’UTR region, fifty-one were found in the intronic region, and two were found in the exonic region. However, only 1 variation was found in the coding region in exon 3. As the PPP1R11 gene was observed to be monomorphic, a single genotype was observed in all the breeding bulls studied. It may be concluded that the study revealed the FSH-β gene was found to be polymorphic in crossbred breeding bulls studied. The PPP1R11 gene showed a restriction cutting site for *Nco*I enzyme, resulting in two fragments of size 332bp and 267bp. One transversion mutation in PPP1R11 gene was observed, where the genotype ‘T’ was replaced by genotype ‘G’ at position 186. The above findings suggest that these variations need to be confirmed for their association with sperm quality traits and fertility in a large population.

SUMMARY

One of the most important traits in animal production is fertility, which is an indicator of successful reproduction. Considering the importance of male fertility, the present investigation was carried out to identify FSH-β and PPP1R11 gene polymorphism in breeding bulls of Maharashtra. The amplified DNA fragments of FSH-β (313bp) and PPP1R1 (599bp) gene were digested using enzymes *Pst*I and *Nco*I, respectively. FSH-β gene digested

with enzyme *Pst*1 produced three different genotype AA (202bp, 99bp), AB (313bp, 202bp and 99bp) and BB (313 bp) with genotypic frequencies of 0.05, 0.16 and 0.78 respectively, whereas the frequency of A and B allele was 0.13 and 0.86, respectively. The PCR product of PPP1R11 was digested with enzyme *Nco*I revealed one cutting site and produced two fragments of 332bp and 267bp in all the animals. While the sequencing results of PPP1R111 revealed a transversion mutation T>G at position 186. The variations in both FSH-β and PPP1R11 genes can be exploited to improve fertility if studied for association with fertility and semen traits.

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