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## Polymorphism study of BMP-15 (FecX<sup>R</sup>) and GDF-9 gene related with twinning in cattle

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**Abstract**

Natural mutations in prolificacy genes in some species especially sheep has been shown transforming growth factor beta (TGF-β) super family ligands such as growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and their type I receptor (bone morphogenetic protein receptor, BMPRI1B) crucial for ovulation and as well as for increasing litter size. Mutations in any of these genes increased prolificacy in carrier. The present investigation was carried out for identification of fecundity genes in cattle, with comparison of DNA fragment between dams bearing twin and singleton and to see inheritance pattern of these fragments in their twin progenies. The animals belonged to six breeds viz. pure (Sahiwal and Hariana) and three crossbreeds (Karan Swiss, Karan Fires, Holstein Friesian) besides other cross breeds of unknown lineage selected from field. The study involved analysis of genome related to twinning using molecular markers (PCR) on BMP-15 (FecX<sup>R</sup>) gene and A competitive technique called tetra-primer amplification refractory mutation system-PCR was adapted to type G1 locus mutation GDF-9 fecundity gene. PCR analysis of the FecX<sup>R</sup> allele of the BMP-15 gene detected the presence of wild-type allele in the dams and their progenies. An allele specific primer used for detection of GDF-9 gene mutation, identified only wild-type and control fragment which revealed the absence of the mutant fragment for this gene locus. The study revealed monomorphic pattern of the two fecundity genes BMP-15 and GDF-9 genes, which has no effect on twinning in cattle.

**Keywords:** Polymorphism, FecX<sup>R</sup> gene, GDF-9 gene, cattle

**Introduction**

Reproduction is the biological process by which new "offspring" are produced from their "parents". Oviparous animals lay eggs, with little or no other embryonic development within the mother. This is the reproductive method of most fish, amphibians, reptiles, all birds, the monotremes, and most insects, some molluscs and arachnids. Viviparous animals retain fertilized eggs inside the body of the female, where they develop (utilizing nutrition of the yolk and albumin) internally until fully independent, young ones are born. Almost all species of mammals are viviparous in their reproduction. One of the most important questions to be considered in the survival of a species is the basis for determining ovulation quota and litter size. The careful regulation of the number of eggs shed and hence the litter size is crucial to successful reproduction in all species (Baird *et al.*, 1998) <sup>[1]</sup>. Cattle (*Bos taurus*) are a uniparous species meaning that, in most cases, females produce only one offspring per pregnancy. Twinning occurs relatively rarely, with the frequency generally not exceeding 1% in most of beef herds, in which selection on this trait has not been practised (Rutledge, 1975) <sup>[22]</sup>. In dairy herds, the incidence of double births is higher (on average 4-5%), and is strongly affected by age and parity of the dam, ranging from about 1% for heifers to nearly 10% for older cows. Twinning rate can increase over a 10-year age of cow period, and the largest increase was observed between the first and second parity (Berry *et al.*, 1994; Cady *et al.*, 1978; Kinsel *et al.*, 1998, Nielen *et al.*, 1989, Ryan *et al.*, 1991) <sup>[5, 6, 16, 18, 23]</sup>. Twinning rate is also slightly influenced by seasonal effects, with a trend toward more multiple births during the spring (Cady *et al.*, 1978; Karlsen *et al.*, 2000) <sup>[6, 14]</sup> or autumn months (Gregory *et al.*, 1990a) <sup>[12]</sup>.

The nature of seasonal effects, although uncertain, is thought to be connected with changes in temperature, the duration of daylight or in feeding at conception. (Fricke *et al.*, 1999; Wiltbank *et al.*, 2000) <sup>[10, 25]</sup> described a direct relationship between high milk production and the increased incidence of double ovulation, which may subsequently result in increased twinning.

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In obstetrics and gynecology, fecundability is the probability of being pregnant in a Single menstrual cycle, and Fecundity is the probability of achieving a live birth within a single cycle (Berek and Novack, 2007) [4]. Twinning is a complex trait with multiple causative factors that include physiological as well as genetic components. Twins are natural experimental models, because each set of twins comes with its own control. Although the mechanism that increases double ovulations in high producing cows remains unknown. The present investigations has been an attempt to identify the fecundity genes (BMP-15 and GDF-9) using PCR method in cattle.

### Material and Methods

The animals investigated in the present study were from the cattle yard of National Dairy Research Institute, Karnal, some dams were selected from the field in Haryana and Punjab, information for which were gathered through communication in newspaper. The details of animal selected given in (Table 1) Experimental animals were selected based on criteria cattle dams which gave birth to twins at least once in their lifetime and twin born individuals one or both members of twin pair available. Control animals were selected based on cattle dams which gave birth to singletons in all calving. Blood samples were collected from the dams and twin born progenies. Aseptically 10-20 ml of blood per animal was collected from jugular vein in separate sterile tubes containing Acid Citrate Dextrose (ACD) @ 1 ml / 6ml blood as an anticoagulant. DNA was isolated from the blood samples using routine standard phenol-chloroform extraction method (Clamp *et al.*, 1993) [4], with some modifications (Shashikant, 1999) [24].

### PCR conditions

Amplification of genomic DNA for identification of the FecX<sup>R</sup> gene polymorphism was carried out by PCR technique using primer FecXR F-(5'- CTCTGAGACCAAACCGGGTA -3') and R-(5'- CATGCCACCAGAACTCAAGA -3'). The primer was selected from already identified gene mutation of BMP-15 gene in Rasa Aragonesa sheep (Monteagudo *et al.*, 2009) [17]. The PCR reaction was set to a volume of 25 µl. The amplification reaction conditions were as follows; initial denaturation at 95 °C for 120 s, followed by 95 °C for 30 s, 57 °C for 30 s, 72 °C for 20 s for 34 cycles and final extension at 72 °C for 5 Min. A 312 bp amplification product was expected in the animals wearing the wild allele for this exon. Another competitive technique called tetra-primer amplification refractory mutation system (T-ARMS) PCR was adapted for screening polymorphism of GDF-9 gene. In tetra-primer ARMS-PCR, four primers are used to amplify a larger fragment from DNA containing the SNP and amplicons representing each of the two allelic forms. Two allele-specific (inner) primers GDF9-I (IFA-5'-CTGCAGCCAGATGACAGAGCTTTTCA-3') (IFG-5'-CGTATGCCTTATAGAGCCTCTTCATGTCGC-3') was designed in opposite orientation and in combination with the common (outer) primers, GDF9-O(OF-5'-GCCTGGCTCTGTTTTCCTATTAGCCTTG-3') (OR-5'-TCTTCTTCCCTCCACCCATTAACCAATC-3') was selected from known literature, so that simultaneous amplification of both the wild-type and the mutant amplicons could be possible (Polley *et al.*, 2010) [20]. The amplification reaction conditions were as follows; initial denaturation at 95 °C for 120 s, followed by 95 °C for 30 s, 57 °C for 30 s, 72 °C for 20 s for 34 cycles and final extension at 72 °C for 5 Min. The resulting amplified products were resolved on a 1.5%

agarose gel horizontal electrophoresis stained by ethidium bromide, gene fragments were visualized on UV Transilluminator and photographed with gel documentation system. The DNA fragments were analyzed by visual comparison of band patterns in the sampled animals.

### Results and Discussion

The process that allows cows to produce two calves rather than one has been of interest for many years. In dairy cattle, twin births have generally been viewed as undesirable due to increased problems in the dam and calf leading to increased costs (Beerepoot *et al.*, 1992, Nielen *et al.*, 1989) [3, 18]. The investigation was conducted on dams and progenies of cattle comprising of two pure breeds (Sahiwal and Harijana) and three crossbreeds (Karan Swiss, Karan Fires, Holstein Friesian) besides other cross breeds of unknown lineage selected from field. Molecular analysis was carried out using PCR and (T-ARMS) PCR for BMP-15 gene and GDF-9 gene respectively on cows, which gave birth to twin at least in one of their calving (experimental), and cows, which gave birth to singletons only (control) and twin born individuals (for inheritance patterns). It involved two aspects and results are presented separately. Firstly, the experimental and control dams were investigated, while in second part the experimental dams and their progenies were studied together to see comparison of banding pattern between the two.

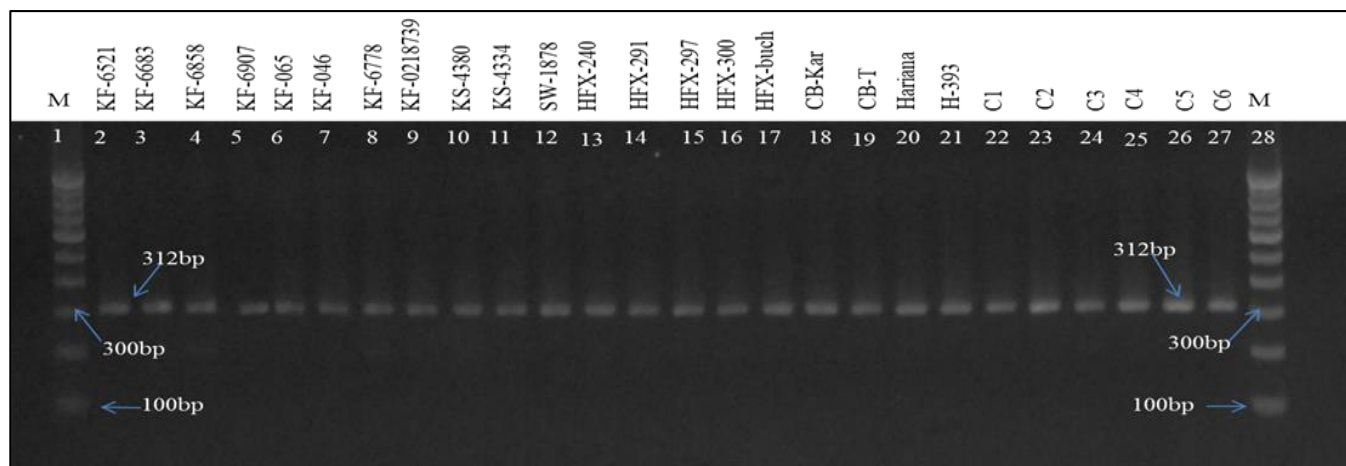
Amplification of genomic DNA of cattle using primer FecXR for BMP-15 gene produced amplicons of 312bp. The amplified products were resolved in 1.5% agarose gel electrophoresis. The cattle dams screened for BMP-15 gene mutation produced product size of 312bp revealing the presence of wild-type allele as shown in (Figure 1). Progeny and dams were compared for band pattern revealed the similar monomorphic bands transmitted a shown in (Figure 2), while the deletion mutation 295bp did not reveal in any of the samples studied here. Bone morphogenetic protein 15 (BMP15) also known as FecX (Galloway *et al.*, 2000) [11] is an X linked gene (FecX locus) of sheep belonging to TGFβ family. The protein product of BMP15, a paracrine factor, stimulates follicle growth, granulosa cell proliferation and cell-survival signaling (Demars *et al.*, 2013) [9]. In homozygous carrier ewes of BMP15, ovarian hypoplasia occurs due to failure of progress of ovarian follicles beyond the primary stage of follicle development resulting sterility (Davis *et al.*, 1992) [8]. BMP15 was found to be associated with prolificacy in Inverdale, Lacaune, Belclare and Small Tailed Han sheep. But, BMP15 regulates ovulation rate and female fertility in a species-specific manner that is indispensable in sheep and largely insignificant in mice (Yoshino *et al.*, 2006) [26]. Monteagudo *et al.*, (2009) [17] reported the wild-type FecX<sup>R</sup> allele of 312bp and mutant allele of 295bp with 17bp deletion of the BMP-15 gene in the Rasa Aragonesa sheep breed, but in this cattle revealed wild-type band of 312bp only.

The tetra-primer ARMS-PCR method was successfully applied to amplify G1 locus GDF-9 gene using primers GDF9-I, GDF9-O produced amplicons of 247bp and 396bp in cattle dams and their progenies, while the mutation pattern of 205bp was not present in any of the animals studied. The amplified products were resolved in 1.5% agarose gel electrophoresis. The first group screened for this mutation revealed amplicons of wild-type fragment 247bp and control fragment 396bp and they were monomorphic as shown in (Figure 3). The second group revealed the same monomorphic

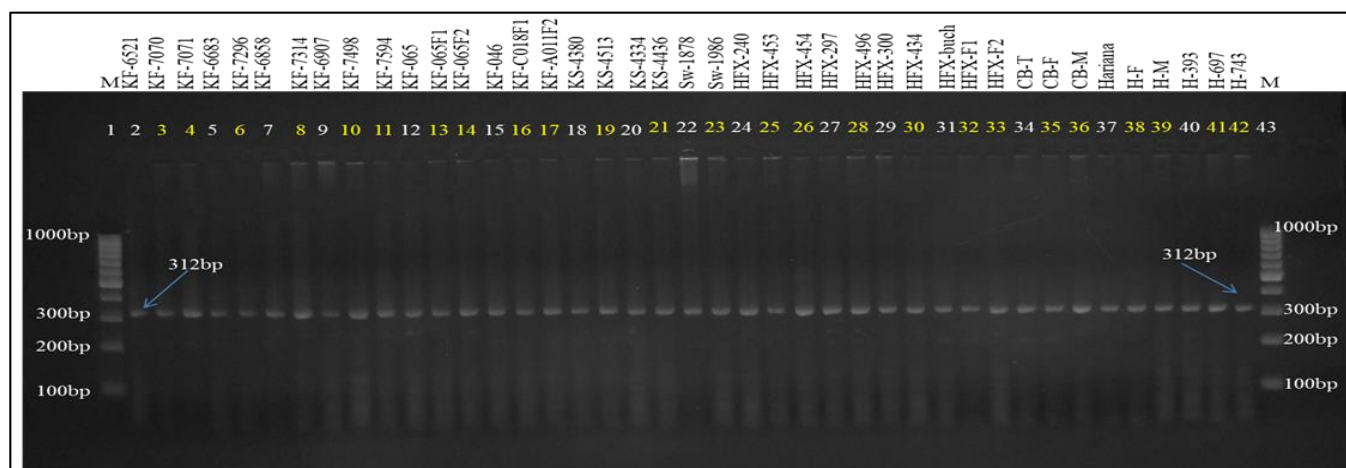
pattern of the wild type allele, thus progenies inherited similar allele from their dams.

Growth differentiation factor 9 (GDF9) also known as FecG is mapped to fifth autosome of sheep and is expressed in oocytes from the primary stage of follicular development until ovulation (Kidder *et al.*, 2010) [15] playing an important role in folliculogenesis (Hanrahan *et al.*, 2004) [13]. One copy of GDF9 increases ovulation rate, but two copies yield streak ovaries and no ovulations. Out of the eight different point mutations (G1-G8) identified in the GDF9, three are

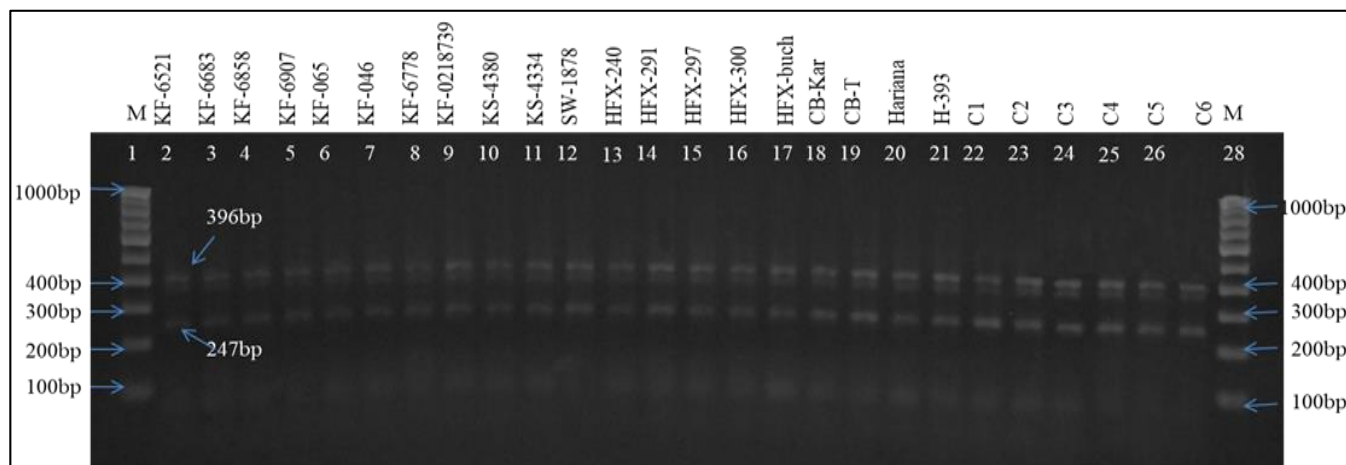
associated with synonymous change in amino acid (G2, G3 and G5) and the remaining five (G1, G4, G6, G7, and G8) are nonsynonymous in Iranian sheep. The six different mutations in FecG have been reported to be associated with fertility in sheep viz. FecGH (G8) mutation in Cambridge and Belclare sheep (Hanrahan *et al.*, 2004) [13]. Polley *et al.*, (2009) [19] revealed wild-type allele of GDF-9 gene in Black Bengal goats similar to the bands seen in cattle as present in this study. Barzegari *et al.*, (2010) [2] reported G1 mutation of GDF-9 gene in



**Fig 1:** PCR product of FeXR gene revealed by 1.5% agarose gel in cattle dams. Lane M: 100 bp DNA ladder, Lane 2-27: PCR product of 312 bp



**Fig 2:** PCR product of FeXR primer revealed by 1.5% agarose gel in cattle dams and twin progenies. Lane 1, 43: 100bp DNA ladder, Lane 2-42: PCR product of 312 bp



**Fig 3:** PCR product of GDF-9 gene revealed by 1.5% agarose gel in cattle dams  
Lane 1, 28- 100 bp DNA ladder,  
Lane 2-27: PCR product Control fragment 396bp, Wild-type fragment 247 bp

Moghani and Ghezel sheep. Polley *et al.*, (2010)<sup>[20]</sup> reported two polymorphism in the Garole sheep; one in GDF-9 gene at the G1 locus with mutant allele having 205bp and wild-type allele having 247 bp, another in BMPR-1B gene with mutant allele 136bp size and wild-type allele 111bp. Roy *et al.*, (2011)<sup>[21]</sup> reported three polymorphism in Bonpala sheep one in the BMPR-1B locus with mutant allele 136bp size and wild-type allele 1110bp, other two in G1 and G4 locus of the GDF-9 gene having 205bp mutant allele and 247bp wild-type allele for G1 locus, 212bp mutant allele and 261bp wild-type allele for the G4 locus. The current investigation studied that the cattle dams and their progenies revealed monomorphic status of the wild type allele *FecX<sup>R+</sup>* and the mutation was absent in the animals sampled. GDF-9 gene locus also showed monomorphic pattern of the wild type allele in the animals studied here, none of them carried mutant allele. In sheep breeds mutations of *FecX<sup>R</sup>* (BMP-15) and GDF-9 gene have been reported to be associated with litter size, however it could not be found in cattle.

### Conclusion

From the study conclusion emerged that the fecundity genes mutations of *FecX<sup>R</sup>* (BMP-15) and GDF-9 gene identified in sheep causing high litter size did not reveal relation with twinning trait in cattle. Polymorphism of BMP-15 and GDF-9 gene mutation was absent in cattle, only the wild type alleles was present. Absence of this gene mutation provides evidence that this fecundity genes has no effect on prolificacy of cattle.

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