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Research Article

COEXISTENCE OF POLYMORPHISM IN FECUNDITY GENES BMPR 1B AND GDF 9 OF INDIAN KENDRAPADA SHEEP

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ABSTRACT: Present study was carried out to find out the status of mutations in three fecundity genes *i.e.* growth differentiation factor 9 (*GDF9/FecG*), bone morphogenetic protein 15 (*BMP15/FecX*) and bone morphogenetic protein receptor (*BMPR1B/FecB*) in Kendrapada sheep, the second most prolific sheep breed of India after Garole. Kendrapada ewes (n=85) were genotyped by Tetra-primer amplification refractory mutation system-PCR and a total of eleven SNP points over these three candidate fecundity genes (one point on FecB and five points each on BMP15 and GDF9) were assessed. Out of eleven, two SNP points, *viz.* FecB and G4 of GDF9 were found to be polymorphic in this breed. In this sheep breed average litter size of the ewes with non-carriers, heterozygous carrier and homozygous carrier of FecB locus mutation were 1.61, 1.80 and 2.06 respectively. G4 point of the GDF9 gene was also polymorphic with average litter size of non-carriers, heterozygous carrier and homozygous carrier ewes were 1.63, 2.00 and 1.91 respectively. This study establishes Kendrapada sheep as the sixth sheep breed after Belclare/Cambridge, Lacaune, Small-tailed Han, Garole and Bayanbulak sheep, where coexisting polymorphism has been found in two different fecundity genes (BMPRIB and GDF9 genes).

Key words: Prolificacy, FecB, FecG, FecX, T-ARMS PCR, Kendrapada sheep, India.

INTRODUCTION

In domesticated sheep (*Ovis aries*), ovulation rate and litter size vary widely among different breeds and within breeds. Although identifying the specific gene(s) behind this complex phenomenon was a formidable task, studies showed that a set of different genes, collectively called fecundity (Fec) genes (Piper and Bindon 1982, Davis *et al.* 1982, Davis 2005) play crucial role in determining ovulation rate and litter size of domesticated sheep. Three major Fec genes, having profound effect on the fecundity in sheep are bone morphogenetic protein receptor type IB (BMPRIB) or activin-like kinase 6 or FecB or Booroola gene (Souza *et al.* 2001), growth differentiation factor 9 (GDF9) or FecG (Hanrahan *et al.* 2004), and bone morphogenetic protein 15 (BMP 15) or FecX (Galloway *et al.* 2000). They are the member of transforming growth factor β (TGF β) super family (Juengel *et al.* 2004, Liu *et al.* 2014).

The BMPR1B gene is present in ovine chromosome 6 and it induces precocious maturation of ovarian follicles that ovulate at smaller size (McNatty *et al.* 1986). A G→A transition at nucleotide position 746 of BMPR1B cDNA produced 'hyperprolific' Booroola sheep, known as FecB (Souza *et al.* 2001, Wilson *et al.* 2001). The mutation in FecB allele is associated with the additive effect on ovulation rate and increase in litter size (Davis 2004, Kumar *et al.* 2006).

Bone morphogenic protein (BMP15) monitors a variety of cellular phenomena including oocyte

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Table 1. Allelic and genotype frequencies of the Kendrapara sheep for *BMPR 1B* locus (FecB) and G4 locus of GDF- 9 gene.

Gene (Mutation)	No. of animal	Allele frequency		Genotype frequency		
		G	A	GG	AG	AA
BMPR-1B (FecB)	85	0.60	0.40	0.353	0.494	0.153
GDF-9 (G4)	85	0.67	0.33	0.58	0.20	0.22

maturation and development (Galloway *et al.* 2002, Shimasaki *et al.* 2004). The gene plays crucial role in regulation of different granulosa cell processes and folliculogenesis. Mutations in the mature *BMP15* coding region have been described (Davis 2005) with a positive impact on prolificacy of various sheep breeds (Hanrahan *et al.* 2004). One copy of the Inverdale (*FecX*^{I}), Hanna (*FecX*^{H}), Belclare (*FecX*^{B}), Galway (*FecX*^{G}) or Lacaune (*FecX*^{L}) allele cause increase in about 0.6 lambs per ewe, compared to the wild type (Hanrahan *et al.* 2004, Bodin *et al.* 2007).

Ovine Growth differentiation factor 9 (*GDF9),* a close autosomal paralogue of BMP15, has been mapped to sheep chromosome 5 (Sadighi *et al.* 2002). GDF9 and BMP15 are supposed to form non-covalent homo and heterodimers *in vivo* and modulate the ovulation rate in species-specific way (Moore *et al.* 2004). Throughout the coding region of the GDF9 gene eight single nucleotide polymorphisms (G1-G8) have been identified (Hanrahan *et al.* 2004). Out of these eight mutation points three points (G2, G3, and G5) are single nucleotide changes that are synonymous in nature. A recent study on GDF9 gene in Garole sheep revealed that 85.71 percent of studied animals had AA genotype while only 14.29 percent animals had AB genotype (Raja *et al*. 2016).

The BMPR1B, together with BMPRII, execute BMP15 signalling in the ovarian follicles (Moore *et al.* 2003). GDF9 and BMP15 have functional significance during cumulus expansion, oocyte maturation and ovulation (Elvin *et al.* 1999, Gui and Joyce 2005, Yoshino *et al.* 2006). Besides these, two paracrine factors control follicle growth (Dong *et al.* 1996, Nilsson and Skinner 2002), cumulus and granulosa cell proliferation (Hayashi *et al.* 1999, Gilchrist *et al.* 2006, Spicer *et al.* 2006), cell-survival signalling (Hussein *et al.* 2005, Orisaka *et al.* 2006) and act as regulator of numerous growth factors and endocrine hormones (Juengel *et al.* 2004). So study to find out existence of polymorphisms in these three important fecundity related genes would be useful to increase the reproductive efficiency of animal through genetic manipulation.

Kendrapada sheep is a prolific breed with its natural abode in coastal districts like Kendrapada, Jagatsinghpur and Cuttack in Odisha, India. Majority of pure bred animals are found in Kendrapada district. The family flock size varies greatly, from as low as 5 to as high as 50. Males and females are maintained in ratio of 1:30 to 1:40. The age of sexual maturity is about 349 days and age at first lambing is about 518 days. The sheep breeds throughout the year but most of them come to heat in summer. Kendrapada sheep produces 35.1% single, 62.6% twin and 2.3% triplet (Patro *et al.* 2006). Recent study by Kumar *et al.* (2008) confirmed presence of FecB mutation in the ewes with increased prolificacy of this breed. It would be interesting to find out whether increased prolificacy of this breed is associated with mutations in other fecundity candidate genes like BMP15 and GDF9, along with FecB.

The objectives of the current study were to detect the polymorphism of GDF9, BMP15 and BMPR1B genes in Kendrapada sheep and to investigate their association with prolificacy of the breed.

MATERIALS AND METHODS

A total of 85 Kendrapada ewes, with a history of multiple births (twins and triplets), were identified from different villages of Kendrapada district, Odisha for our study. About 10 ml of venous jugular blood sample was collected from each animal in sterile vacutainer containing K_3-EDTA as an anticoagulant. The collected blood samples were stored at 4°C and transported to the laboratory at the earliest. After processing, the genomic DNA was isolated from white blood cells using standard phenol-chloroform procedure (Sambrook and Russell 2001). The DNA samples were dissolved in TE buffer (pH 8.0) and stored at -20ºC before use.

Tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR), as described by Ye *et al.* (2001), was carried out to detect the FecB point mutation of BMPR1B gene. Briefly two pairs of primers were designed to amplify fragments of differing sizes, each representing one allele. Amplicons were easily resolved by standard agarose gel electrophoresis. A total of ten point mutations of other two important candidate genes, five each in GDF9 (G1, G4, G6, G7 and G8) and BMP15 (FecX^{G}, FecX^{H}, FecX^{I}, FecX^{L} and FecX^{B}) gene

Table 2. Effect of different genotype of FecB and G4 on lambing size.

Gene	SNP	Genotype	No. of animals	Average litter size
B M P R- IB		AA	1 3	1.61
	Fec B	AG	4 2	1.80
		G G	3 0	2.06
G D F 9		AA	1 9	1.63
	G 4	AG	1 7	2.00
		G G	4 9	1.92

were screened by T-ARMS-PCR as mentioned above using the primers described earlier (Polley *et al.* 2009, 2010).

PCR reaction was performed in a 25 µl reaction mixture containing 2.5µl of 10× Paq5000 reaction buffer (provides a final Mg^{+2} concentration of 2mM), 200 µM of dNTPs (Fermentas, Lithuania), 5 pmol of each primer and 0.5 units of Paq5000™ DNA polymerase (Stratagene, USA). About 50 ng of genomic DNA was used as template. The PCR cycling parameters were optimized separately for detecting each of the allele specific amplifications. The PCR products were separated by horizontal submarine agarose gel (2-3%, free from DNAse and RNAse, Sigma, USA) electrophoresis in 1× TAE buffer at 100 V. The gel was stained with ethidium bromide solution (0.5µg/ml), maintained for 10 min. in darkness and photographed using a molecular imager (Gel Doc XR, BIORAD).

RESULTS AND DISCUSSION

The molecular crosstalk among BMP15, BMPR1B and GDF9 and their liaison with female fecundity in domesticated sheep is a well-acquainted fact. Recently, G to A nucleotide substitution was found in GDF9 locus that affects litter size in Iranian Baluchi sheep (Moradband *et al*. 2011). Two mutations including *BMPR-IB/FecB* and *GDF9/G1* were also found in Bayanbulak sheep (Zuo *et al*. 2013).

BMPR1B: In BMPR1B gene, 'A' allele (non-carrier) represents the wild type and 'G' allele (carrier) represents the mutant type (FecB). Genomic DNA samples from 85 Kendrapada sheep were analyzed and all three possible genotypes, AA, AG and GG were observed. These three genotypes (AA, AG and GG) represent the wild homozygous, mutant heterozygous and mutant homozygous respectively. Presence of each genotype was confirmed by visualizing amplicon size variation following PCR. Amplicon sizes of 1178 bp (common outer) and 1097 bp ('A' allele specific) confirmed AA genotype. In case of mutant carrier 'GG' sheep 1178bp and 136 bp ('G' allele specific) products were amplified. However, in heterozygous sheep 1178 bp, 1097 bp and 136 bp products were amplified (Fig. 1). The overall genotype frequencies of AA, AG and GG were found to be 0.153, 0.494 and 0.353 respectively. The allelic frequencies for A and G alleles were 0.40 and 0.60

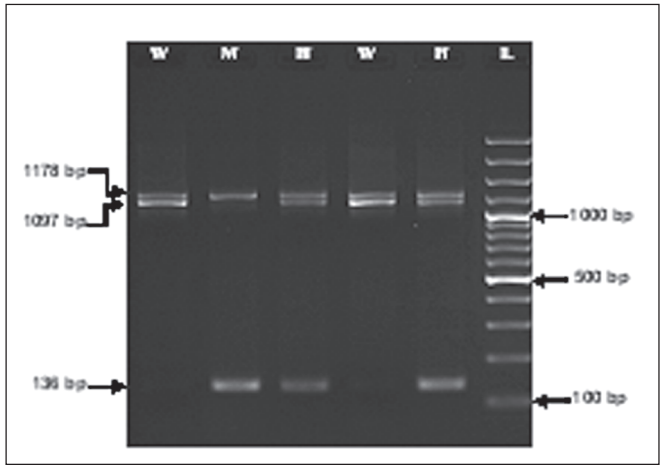

Fig. 1. Agarose gel electrophoresis (2%) of tetra-primer amplification refractory mutation system-PCR amplicons for FecB locus of BMPR1B gene.

The size of common outer product (1178 bp) and allele-specific wild-type (1097 bp) and mutant-type (136 bp) inner product are indicated by arrow on the left side of the gel photograph. Lane L: DNA molecular weight marker (O'RangeRular 100bp DNA Ladder, Fermentas, Lithuania) indicated by arrow on the right side of the gel photograph.

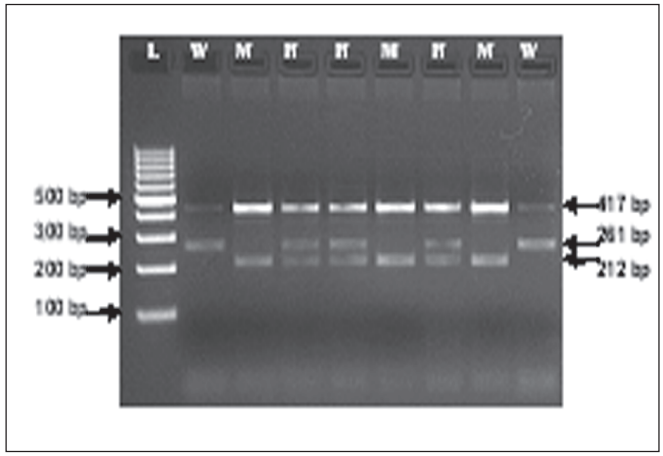

Fig. 2. Agarose gel electrophoresis (2.5%) of tetra-primer amplification refractory mutation system-PCR amplicons for G4 locus of GDF9 gene.

The size of common outer product (417 bp) and allele-specific wild-type (261 bp) and mutant-type (212 bp) inner product are indicated by arrow on the right side of the gel photograph. Lane L: DNA molecular weight marker (O'RangeRular 100bp DNA Ladder, Fermentas, Lithuania) indicated by arrow on the left side of the gel photograph.

respectively (Table 1). The results indicate that about 85% of the individuals in the study population are carrying mutation. Frequency of the mutant type allele is more than the wild type allele frequency (0.6 vs. 0.4) but is less than 0.73, determined earlier by Kumar *et al.* (2008) in the same breed in smaller sized flock. Average litter sizes of non-carrier wild types, heterozygous and mutant ewes were 1.61, 1.80 and 2.06 respectively (Table 2). The lambing records of the experimental sheep reveals that average litter size is the highest in homozygous mutant type followed by heterozygous mutant and homozygous wild type animals. This finding is in line with the other findings related to the phenotypic effect of FecB mutation in sheep. This is the sixth sheep breed in the world after Booroola, Garole, Javanese, Hu and small tailed Han carrying the particular point mutation that has impact on the litter size (Kumar *et al.* 2008).

BMP15: Earlier studies pointed out that the ewes with mutations in five different points ($FecX^{I}$, $FecX^{H}$, Fec X^{L}, $FecX^{G}$ and $FecX^{B}$) of BMP15 gene showed increased ovulation rate in heterozygous condition and sterility in homozygous condition due to impaired follicular development (Davis *et al.* 1982, Bodin *et al.* 2002, Hanrahan *et al.* 2004). In the current study, same five point mutations (Q239ProTer, Q23Ter, V31D, C53Y and S99I) in BMP15 were typed by tetra-primer ARMS-PCR in Kendrapada sheep. However, in our study, we did not find mutation in any of these five points. It implicates that increased litter size in breed has no association with the five known SNPs of BMP15. Several instances are there where no BMP15 mutations were found in these points in the different prolific sheep breeds of the world. None of Small Tailed Han and Hu sheep carried the $FecX^{B}$ or $FecX^{H}$ mutation (Liu *et al.* 2003, Chu *et al.* 2005[a,b]). None of Garole, Javanese, Small Tailed Han, or Hu sheep had the Fec X^{I} mutation (Davis *et al.* 2002, Liu *et al.* 2003, Chu *et al.* 2005[a]). The prolific Hu sheep did not have the $FecX^{G}$ mutation (Chu *et al.* 2005[a]). The present report is also in agreement with the recent finding of Kumar *et al.* (2008) that Kendrapada sheep with high litter size and twinning rate is not carrying $FecX^{G}$ mutation.

GDF9: Only G4 point was found to be polymorphic in Kendrapada sheep after screening five point mutations in GDF9 gene at five positions (G1, G4, G6, G7 and G8) (Fig. 2). Hanrahan *et al.* (2004) reported that change in a specific point mutation in GDF9 gene (G8) causes an amino acid substitution that leads to increased prolificacy in Belclare and Cambridge sheep. Ewes with heterozygous GDF9 (G8) mutation have an increased ovulation rate but infertile in case of homozygous mutant (Juengel *et al.* 2004). In the present study, both 'A' and 'G' alleles were detected in G4 locus with frequencies of 0.33 and 0.67 respectively. All three possible genotypes were observed (AA, AG and GG) with genotype frequency of 0.22, 0.20 and 0.58 respectively (Table 1). The mutant (GG) genotype of G4 point was found to be the predominant genotype in tested Kendrapada population. Our results showed that ~80% of the individuals under study were carrying mutation in the G4 point of the gene. Average litter size of these three genotypes non-carriers wild types, heterozygous and mutant ewes had 1.63, 2.00 and 1.91 respectively (Table 2). Here is an interesting finding that presence of single copy of mutant allele in the G4 SNP point of GDF9 gene *i.e.*, in the heterozygous mutant animal shows maximum average litter size. Available literature suggests partly similar kind of observation in case of GDF 9 gene and conferred the phenomenon as a case of heterozygote advantage (Dong *et al.* 1996, Gemmell and Slate 2006). However, consolidated data collected over many years on ovulation rates, litter size and lambing rates of this local sheep breed is required to conclude the present case as compelling example of heterozygote advantage.

CONCLUSION

Finally, the results indicate that among 11 SNP points on three fecundity genes, at least two loci, FecB loci of BMPR1B gene and G4 of GDF9, are polymorphic in the Kendrapada sheep. Therefore, Kendrapada sheep is the sixth sheep breed after Belclare/Cambridge, Lacaune, Small-tailed Han, Garole and Bayanbulak where coexisting polymorphism has been found in two different fecundity genes. Further studies are needed to draw conclusion on the origin of these mutations and whether it is influenced by Garole or some other local breeds. Profitable and sustainable sheep production through genetic manipulation is a high priority for India. Reproductive performance of non-prolific local sheep breeds of India carrying other desired traits can be optimized through incorporation of prolificacy genes by advanced breeding strategy. Therefore, apart from Garole, Kendrapada may be the breed of choice for improving the prolificacy of Indian sheep breeds.

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