



Association of Polymorphisms in Fecundity Genes of GDF9, BMP15 and BMP15-1B with Litter Size in Iranian Baluchi Sheep

F. Moradband, G. Rahimi* and M. Gholizadeh

Laboratory for Molecular Genetics and Animal Biotechnology, Department of Animal Sciences,
Faculty of Animal and Aquatic Sciences, Sari Agricultural Sciences and Natural,
Resources University, Sari, Iran

ABSTRACT : The incidence of mutation in three loci of GDF9, BMP15 and BMP15-1B and their effects on litter sizes was evaluated in Baluchi sheep. Wild-type alleles were detected for BMP15 and BMP15-1B loci and all individuals were found to be as non-carriers for *FecB* and *FecX^G* mutations but, a G to A nucleotide substitution was found in GDF9 locus. The frequency of *FecG⁺* (0.82) wild type allele was higher than the frequency of *FecG^I* (0.18) mutant allele and the frequencies of *FecG⁺/FecG⁺*, *FecG⁺/FecG^I* and *FecG^I/FecG^I* genotypes were 0.72, 0.20 and 0.08, respectively in GDF9 locus. The heterozygous (*FecG⁺/FecG^I*) and homozygous (*FecG⁺/FecG⁺*) non-carrier ewes had 0.35 and 0.21 more lambs than the homozygous (*FecG^I/FecG^I*) carrier ewes, respectively ($p < 0.05$). In addition to the finding of segregation of non-additive gene effect on litter size in the previous study in Baluchi sheep, these findings for the first time shows that the *FecG^I* gene has a major effect on litter size in this breed. (**Key Words :** BMP15, BMP15-1B, GDF9, Litter Size, Baluchi Sheep)

INTRODUCTION

Research conducted since 1980 in relation to inheritance patterns and DNA testing of major genes for prolificacy has shown that major genes have the potential to significantly increase the reproductive performance of sheep flocks throughout the world (Davis, 2005). In sheep, three prolificacy loci have been discovered, namely bone morphogenetic protein receptor type 1B (BMPR1B; or activin-like kinase 6, ALK6), known as *FecB* (Booroola) on chromosome 6 (Souza et al., 2001) corresponding to the human chromosome 4q22-23 (Montgomery et al., 1993); growth differentiation factor 9 (GDF9), known as *FecG* on chromosome 5 (Hanrahan et al., 2004); and bone morphogenetic protein 15 (BMP15) known as *FecX* on chromosome X (Hanrahan et al., 2004; Galloway et al., 2000). In sheep, 6 different point mutations (*FecX^I* (Inverdale), Galloway et al., 2000; *FecX^H* (Hanna) Galloway et al., 2000; *FecX^L* (Lacaune), Bodin et al., 2003; *FecX^G* (Galway), Hanrahan et al., 2004; and *FecX^B* (Belclare), Hanrahan et al., 2004) and a 17 bp deletion of

the functional gene (*FecX^R*) in Rasa Aragonesa sheep breed (Monteagudo et al., 2009) have been identified in the BMP15 gene, each having a major effect on prolificacy. Ewes with two inactive copies of the BMP15 gene (homozygous animals) are sterile (Galloway et al., 2000; Hanrahan et al., 2004) and have a similar ovarian phenotype. Ewes with a single inactive BMP15 gene (heterozygous animals) are fertile and have an increased ovulation rate and a higher incidence of twin or triplet births (Davis et al., 1991, 1993; Galloway et al., 2000; Hanrahan et al., 2004). Eight different point mutations (G1-G8) have been identified in the GDF9 gene. Three out of the eight polymorphisms are nucleotide changes that do not result in an altered amino acid (G2, G3 and G5). The five remaining nucleotide changes, G1, G4, G6, G7, and G8, give rise to amino acid changes. The G1 arginine to histidine change at amino acid residue 87 in exon 1 substitutes one basic charged polar group with another and occurs at a position before the furin cleavage site for the mature peptide, so is unlikely to affect the activity of the mature protein (Hanrahan et al., 2004). Ewes with a single copy of the mutated GDF9 (*FecG^H*) gene are fertile and have an increased ovulation rate (Hanrahan et al., 2004). In contrast, ewes homozygous for this mutation are infertile with

* Corresponding Author : G. Rahimi. Tel: +98-911-1513087,
Fax: +98-151-3822577, E-mail: rahimimianji@yahoo.com
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primary ovarian failure. Ewes heterozygous for mutations in both GDF9 and BMP15 are fertile and the effects of these mutations on ovulation rate are additive (Hanrahan et al., 2004). The *FecB* gene is a dominant autosomal gene responsible for the fecundity of Booroola Merino sheep with an additive effect on ovulation rate firstly identified in 1980s (Piper et al., 1985; Davis, 2004). In *FecB* animals, a single A to G substitution at nucleotide position 830 results in an arginine replacing a glutamine amino acid in a highly conserved region of this receptor (Hua and Yang, 2009). Ewes inheriting one copy of the *FecB* gene from either parent produced about 1.5 extra eggs and gave birth to about 1.0 extra lamb per ewe lambing. Homozygous carriers produced about 3.0 extra eggs resulting in about 1.5 extra lambs per ewe lambing (Davis, 2004). Baluchi sheep is the largest breed in Iran. This is a fat-tail breed well adapted to a wide range of harsh environmental conditions in eastern Iran, one of the arid subtropical areas of the world. The fleece is white with pigmented head and legs. The wool is coarse with modulation. Body size varies between 35 and 40 kg in adult ewes, milk yield between 40 and 50 kg in a period of about 125 days and annual greasy fleece weight between 1.3 and 1.8 kg. Over 30 years of selection for increased litter size in Baluchi sheep of Abbasabad Sheep Breeding Station did not result in any significant genetic trend in this trait due to very low narrow-sense heritability. Low heritability of litter size in Baluchi sheep indicates that additive genetic variance is not a major component of the phenotypic variance. Saneii and Nejati-Javaremi (2000) have used over ten thousand records of litter size in Baluchi sheep to estimate component of variance based on Gibbs sampling approach with a mixed major gene-polygenic model. Their study indicates the presence of an over-dominant autosomal major gene affecting litter size in Baluchi sheep. Molecular genetic studies would help further understanding of this major gene and its biology. Reports on major genes affecting litter size in other breeds of sheep justify the search for single genes of major effect in this breed of sheep. The aim of this study was to investigate the presence of polymorphism in BMP15 (*FecX^G*), BMP15-1B (*FecB*) and GDF9 (*FecG^I*) and their possible association with litter size in the Baluchi sheep breed.

MATERIALS AND METHODS

Sample collection and DNA isolation

Venous jugular blood samples (10 ml per ewe) were collected from 152 Baluchi sheep breed, along with data on litter size in the first, second, third or fourth parity (Abbasabad Sheep Breeding Station, located northeast of Mashhad, Iran). The collected blood samples were transferred to the laboratory using cooling chain and stored at -20°C for further analysis. Genomic DNA was isolated

by standard salting-out procedure described by Miller et al. (1988). The quality and quantity of the extracted DNA was checked by spectrophotometer and agarose gel electrophoreses. DNA samples were adjusted to a concentration of 100 ng/μl and exactly 1 μl of the DNA samples were used as template for polymerase chain reaction.

Single nucleotide polymorphism detection assays

The G to A nucleotide change (G1) in exon 1 of GDF9 gene was carried out by RFLP-PCR as described by Hanrahan et al. (2004). The primers were designed as follows: (G9-1734F: 5'-GAAGACTGGTATGGGGAAATG-3' and G9-2175R: 5'-CCAATCTGCTCCTACACACCT-3'). The amplification reaction conditions were carried out using 35 cycles at 94°C for 5 min., followed by 94°C for 45 s, 58°C for 40 s, 72°C for 1 min and final extension at 72°C for 10 min. The 462 bp PCR products were digested at 37°C for 3 h with *HhaI* restriction enzyme. Analyses of samples for BMP15 (*FecX^G*) mutation was screened using the forced RFLP method describe by Hanrahan et al. (2004). A specific primer pair has been designed to generate a forced *HinfI* mutation (C to T nucleotide change) in the BMP15 gene, whereas products from non-carriers of the mutation lack this site. Genomic DNA was amplified using primer sequences as: (B2-*HinfI*F: 5'-CACTGTCTTCTTGTTACTGTATTC AATGAGAC-3' and B26: 5'-GATGCAATACTGCCTG CTTG-3'). The amplification reaction conditions was carried out using 35 cycles at 94°C for 5 min, followed by 45 s, 62°C for 40 s, 72°C for 45 s, followed by 72°C for 10 min. The 141 bp PCR products were digested with *HinfI* restriction enzyme. The *FecB* genotyping was carried out using forced RFLP-PCR technique with the following primer sequences *FecBF*: 5'-CCAGAGGACAATAGCAA AGCAAA-3' and *FecBR*: 5'-CAAGATGTTTTTCATGCCTC ATCAACACGGTC-3' as described by Davis et al. (2002). The reverse primer was introduced a point mutation resulting in PCR products with *FecB* carrier sheep containing an *AvaII* restriction site, whereas products from non-carriers of the mutation lack this site. The amplification reaction conditions were carried out using 30 cycles at initial denaturation at 94°C for 5 min, followed by 94°C for 15 s, 60°C for 30 s, 70°C for 30 s and final extension at 72°C for 5 min. The forced PCR of the *FecB* gene produced a 190 bp band. The PCR products were digested at 37°C over night with *AvaII* restriction enzyme. All digested PCR products from three marker loci were separated by 3.0% agarose gel and visualized with ethidium bromide staining under gel documentation system.

Statistical analysis

A χ^2 test for goodness-of-fit was performed to verify if genotype frequencies agreed with Hardy-Weinberg

equilibrium expectations. The following fixed effects model was employed for analysis of litter size in Baluchi ewes and least squares mean was used for multiple comparisons in litter size among different genotypes.

$$Y_{ijk} = \mu + P_i + G_j + e_{ijk}$$

Where Y_{ijk} is the phenotypic value of litter size, μ is the population mean, P_i is the fixed effect of the i^{th} parity ($i = 1, 2, 3, 4$), G_j is the fixed effect of the j^{th} genotype and e_{ijk} is the random residual effect of each observation. Analysis was performed using the GLM procedure of SAS (2002) program.

RESULTS

The 134 Baluchi ewes which had least one record for litter size were screened for GDF9, BMP15 and BMP15-1B mutations in the present study. A DNA fragment with the expected size of 462 bp was amplified from exon I of GDF9 gene. The G to A nucleotide change in GDF9 exon I disrupts a *HhaI* restriction enzyme cleavage site (GCGC to GCAC) at nucleotide 260 of the 462-bp PCR fragment produced by primers G9-1734 and G9-2175. Restriction digestion of the PCR product from wild-type animals with *HhaI* resulted in cleavage of the 462-bp product (at two internal *HhaI* sites) into fragments of 52, 156, and 254 bp ($FecG^+$). But, DNA fragments containing the A nucleotide yield only two fragments of 52 and 410 bp ($FecG^I$). Animals heterozygous for the mutation have fragments of all four sizes (52, 156, 254, and 410 bp). All three possible genotypes were observed in Baluchi sheep population (Figure 1). The allele and genotype frequencies of GDF9 gene was presented in Table 1. For the GDF9 gene, allele $FecG^+$ had the highest frequency (0.82), whereas allele $FecG^I$ had the lowest frequency (0.18) in Baluchi sheep flock. The Genotype frequencies of $FecG^+/FecG^+$, $FecG^+/FecG^I$ and $FecG^I/FecG^I$ were 0.72, 0.20 and 0.08, respectively (Table 1). The BMP15 ($FecX^G$) mutation was analyzed in Baluchi sheep and all of ewes were found non-carriers for the $FecX^G$ mutation. Point mutation in BMP-1B

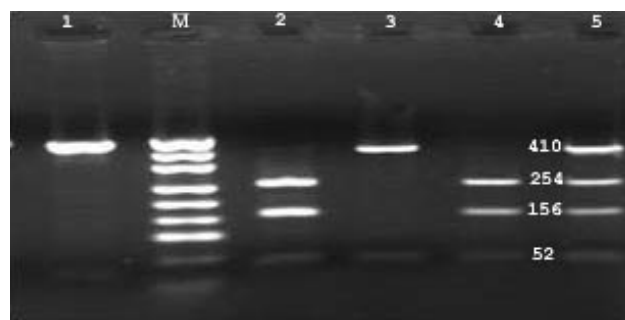


Figure 1. Agarose gel electrophoresis (3%) of digested product of GDF9 gene exon I. Lane 1: PCR product (462 bp). Lane 2: Molecular weight marker. Lanes 3 and 5: Wild type genotypes ($FecG^+/FecG^+$), Lanes 4: Mutant genotype ($FecG^I/FecG^I$). Lane 6: Heterozygous genotype ($FecG^+/FecG^I$).

(*FecB*) was typed by RFLP-PCR and all of individuals found non-carriers for this mutation. The results of variance analysis for litter size in Baluchi sheep are summarized in Table 2. Litter size was significantly influenced by parity ($p < 0.05$) and GDF9 genotype ($p < 0.01$). The least squares means and standard error for litter size of different GDF9 genotypes and parities in Baluchi sheep are given in Table 3. The Baluchi ewes with heterozygous ($FecG^+/FecG^I$) and homozygous wild-type ($FecG^+/FecG^+$) genotype had 0.35 and 0.21 ($p < 0.05$) lambs more than the homozygous ($FecG^I/FecG^I$) genotypes, respectively.

DISCUSSION

PCR-RFLP is a rapid, simple and exact technique for single nucleotide polymorphism (SNP) genotyping. This approach has been used previously to genotype prolific sheep and goat by several research groups (Souza et al., 2001; Davis et al., 2002; Kumar et al., 2006; Guan et al., 2007; Polley et al., 2009). In our experiment, a forced PCR-RFLP approach was used to detect the genotype based on the method described by Hanrahan et al. (2004) and Davis et al. (2002). The results of our study showed that the investigated mutations of $FecX^G$ and $FecB$ that have a major effect on litter size are not present in Baluchi sheep. These results are in agreement with reports in Romanov, Finn,

Table 1. Allelic and genotype frequencies of GDF9 gene in Baluchi sheep population

Gene	No of animals	Allele frequency		Genotype frequency		
		$FecG^+$	$FecG^I$	$FecG^+/FecG^+$	$FecG^+/FecG^I$	$FecG^I/FecG^I$
GDF9	134	0.82	0.18	0.72	0.20	0.08

Table 2. Analysis of variance for litter size in Baluchi sheep population

Source of variation	SM	DF	MS	F-value	Pr>F
Parity	2.163	3	0.721	3.34*	0.019
Genotype	2.755	2	1.377	6.37**	0.0019

SM = Sum of squares, DF = Degree of freedom, MS = Mean of squares. * $p < 0.05$, ** $p < 0.01$.

Table 3. Least squares mean and standard error for litter size of different GDF9 genotypes and parities in Baluchi sheep

Variable		Litter size
Genotype	<i>FecG</i> ⁺ / <i>FecG</i> ⁺	1.238±0.032 ^a
	<i>FecG</i> ⁺ / <i>FecG</i> ^I	1.386±0.051 ^a
	<i>FecG</i> ^I / <i>FecG</i> ^I	1.032±0.094 ^b
parity	1	1.272±0.048 ^a
	2	1.309±0.048 ^a
	3	1.247±0.0652 ^a
	4	1.0467±0.0786 ^b

^{a,b} Least squares means with the different superscripts differ significantly ($p < 0.05$).

East Friesian, Teeswater, Blueface Leicester, D'Man, Chios, Mountain Sheep, German Whiteheaded Mutton, Lley, Loa, Galician, Barbados Blackbelly (Davis et al., 2006), Mulpura sheep (Kumar et al., 2006), Suffolk, Dorset, Charolais, Chinese Merino and Romney Hills sheep (Guan et al., 2007), where *FecB* mutant allele do not segregate in these breeds. In contrast, the presence of *FecB* mutation is reported in several sheep breeds such as Chinese HU and Han sheep breeds (Yan et al., 2005; Davis et al., 2006; Guan et al., 2006), Australian Booroola Merino (Souza et al., 2001; Wilson et al., 2001), Indian Garole (Davis et al., 2002), and Javanese sheep of Indonesia (Davis et al., 2002). In the present study, tests were carried out only for the Galway *FecX*^G mutation and we did not find any genetic variations within the BMP15 gene by PCR-RFLP among the individuals of the Baluchi sheep. These mutations include nonsynonymous amino acid substitution (*FecX*^I, *FecX*^B and *FecX*^L), premature stop codons (*FecX*^G, *FecX*^H) and a 17 bp deletion of the reading frame of the functional gene (Monteagudo et al., 2009). It has been reported that the *FecX*^G mutation of *BMP15* causing infertility among homozygous ewes has been identified in Cambridge and Belclare breeds (Hanrahan et al., 2004). The polymorphism behavior of both Booroola and Inverdal genes were analyzed as likely candidate genes influencing high prolificacy in Luri-Bakhtiari and Shal sheep breeds of Iran but no polymorphism has been found in these breeds (Amiri et al., 2008; Ghaffari et al., 2009). However, the analysis of polymorphism for *FecB* and *FecX*^G loci in Baluchi sheep indicates that the genetic factor responsible for twinning or multiple lambing rates is not related to the reported mutated alleles at the *FecB* and *FecX*^G major genes in this breed. Therefore, we should attempt to detect other SNP for these genes and/or other loci responsible for twinning rate in this breed. In contrast with Hanrahan et al. (2004), our preliminary results in the present study showed that the point mutation in GDF9 gene (*FecG*^I) might be a major gene that influences prolificacy in Baluchi sheep. The *FecG*^I led to increased litter size in heterozygous animals

(1.386±0.050) compared to wild type genotype (1.238±0.032) and homozygous carrier ewes (1.032±0.094) in Baluchi sheep. The G1 arginine to histidine change occurs at amino acid residue 87 in exon 1 and this substitution may likely affect the activity of mature GDF9 protein. Although both arginine and histidine are basic polar amino acids, they exhibit different ionization rate under various physiological conditions. Arginine has a pK_a of 12.5 which essentially always has protonated side-chains at physiological pH. But histidine has a pK_a that varies over a relatively wide range, because its pK_a depends heavily on the local environment. Histidine can act as a pH-dependent switch in proteins, by forcing changes in the protein structure as a result of the additional charge introduced by the proton (Brant, 2010). This is the first report demonstrating that the mutation in GDF9 gene (*FecG*^I) is associated with litter size in sheep. Among the eight point mutations (G1-G8) which had been found in GDF9 gene, only one mutation (G8: *FecG*^H) was associated with increased ovulation rate in heterozygous carriers and sterility in homozygous carriers in Cambridge and Belclare sheep (Hanrahan et al., 2004). In a previously study, the segregation of an autosomal major gene with over-dominance inheritance affecting litter size has been found in Baluchi sheep using Gibbs sampling approach with a mixed major gene-polygenic model (Saneai and Nejati-Javaremi, 2000). In our study, results within the Baluchi sheep flock showed that mean litter sizes of ewes with homozygous wild-type (*FecG*⁺/*FecG*⁺) and heterozygous (*FecG*⁺/*FecG*^I) genotypes were not statistically different whereas these ewes had a higher litter size than homozygous mutant (*FecG*^I/*FecG*^I) genotype ($p < 0.01$). Although, the mode of gene action in these two research works are different, both of these studies support the lack of additive mode of gene action and segregation of major gene in Baluchi sheep breed.

CONCLUSION

Identifying genes of major effect offers the opportunity to improve production efficiency, product quality and product diversity in livestock industry, through utilizing them in breeding programs. The results of this study show that *FecX*^G and *FecB* mutations are not present among this selected population of Iranian Baluchi sheep breed. Preliminary polymorphism analysis performed on *FecG*^I mutation in GDF9 locus suggested a major gene inheritance of prolificacy in this selected population. In conclusion, we cannot completely exclude the possibility that the observed effects could be caused by linkage disequilibrium with other functional SNP in a nearby regions of GDF9 gene or by linkage disequilibrium with other nearby loci.

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