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Research article

Identification of different allelic forms in exon 1 of FSHβ in Iranian Baluchi and Naeini sheep breeds by PCR-SSCP

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Abstract

Follicle-stimulating hormone (FSH) is fundamental for gamete maturation and is known to regulate the production of several growth factors that play a critical role in primordial follicle activation and growth. The aim of this study was to evaluate the exon 1 polymorphism of FSHβ gene in Naeinian and Baluchi sheep breeds. Blood samples were randomly collected from 109 both of two sheep breeds (70 Naeinian sheep and 39 Baluchi sheep). DNA was extracted using modified salting out method. A fragment of 220 bp from exon 1 region was amplified using a pair of specific primers by polymerase chain reaction (PCR). In order to detection of allelic different forms were used from PCR-SSCP and direct sequencing techniques. PCR-SSCP analysis demonstrated eight different banding patterns from exon 1 region of FSHβ gene. After genetic analysis on sequencing results and comparing with the sequence of ovine FSHβ published on GeneBank (accession number S64745.1), a nucleotide insertion in non-coding region of FSH (110 A-T→ACT) was detected. The obtained results from both of two techniques revealed that this region has different allelic forms.

Keywords: PCR-SSCP; FSHβ; polymorphisms; Baluchi; Naeinian

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Introduction

One of the most important economical traits of sheep is litter size. The increase of number of lamb per a year has provided a good chance to improve meat production efficiency (Dickerson 1970; Shelton, 1975). Several attempts have proved the existing of major genes for twining traits. Recently, a new breed of Merinos' sheep, Booroola, has been identified in Australia and New Zealand that have twining traits. A previous study concluded that the reproductive traits in sheep can be controlled by major and minor genes simultaneously (Davis et al., 2006).

Ovulation rate is determined by complex exchange of hormone signals between pituitary and ovary, and local exchange of hormones in ovarian follicles between oocyte and flanking somatic cells. As there is a lot of variation in ovulation rate among different sheep breeds in world, sheep is consider as a good model to evaluate growth, follicle selection and genetic control of ovulation rate. So far, several major genes effective in ovulation rate in sheep have been identified (Davis et al., 2006). In addition, previous studies showed several mutations in BMPR-1B, BMP15 and GDF9 can increase the ovulation rate in sheep (Fogarty, 2009). In 1980, FecB gene was identified as first major gene associated with fertility trait. Studies have showed that fertility rate and twining are controlled by different gene cluster known as Fec genes (Davis et al., 1982; Piper & Bindon, 1982). So far, researchers reported Studies have showed that fertility rate and twining are controlled by different gene cluster known as Fec genes (Davis et al., 1982; Piper & Bindon, 1982). So far, researchers reported three major gene for fertility in

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sheep including BMPR-1B (FecB) on chromosome 6, GDF9 (FecG) on chromosome 5 and BMP15 (FecX) on chromosome X (Piper & Bindon, 1982).

Follicle stimulating hormone (FSH) is a pituitary-expressed glycoprotein hormone and its role is to regulate reproductive traits in mammalian species (McLachlan et al., 1995; Ohta et al., 2007). In male, FSH and testosterone are critical hormones to regulate Sertoli cell function and FSH is necessary for preserving quantity and quality of sperms in spermatogenesis process (McLachlan et al. 1995). Also, the role of this hormone in female revealed that defective secretion of this hormone led to infertility and smaller ovules. FSH is composed of two subunits, the common alpha subunit and hormone-specific beta subunit. Although both subunits play role in connecting the FSH receptor, FSHβ determines the specificity of this connection (Chen et al., 2001). FSHβ gene is located in chromosome 15 in sheep and includes three exons and two introns. Polymorphism of this gene may change its expression and have effect on regulation and concentration of FSH consequently. The mentioned changes can affect ovulation rate and fertility response (Chen et al., 2001). Recently, a 280 bp genetic element in intron 1 of this gene was significantly associated with litter size in pigs (Liu et al., 2009). A study showed that two polymorphism (G40A and T148C) in exon 2 were associated with litter size in Xinong and Boer goat breeds (Bindon et al., 1979). FecB carriers have been found to carry higher FSH concentration (Hunter et al., 2004). Zhang et al. (2011) evaluated polymorphisms in exon 1 and 3 of FSHβ gene in goats and showed polymorphisms in exon 3 associated with reproductive traits.

Another study indicated that SNP (Single Nucleotide Polymorphism) is significantly associated with litter size (Li et al., 2011). So far, a few studies have reported the effect of genes on reproductive traits in Iranian sheep and goats. Recently, Nazifi et al. (2015) revealed polymorphisms of exon 2 in both of Baluchi and Iran Black sheep breeds using PCR-SSCP methods. The evaluation of candidate genes for lambing rate in Iranian sheep showed that the effective candidate genes for fertility rate which is reported in other foreign sheep in world, were not observed in the Iranian sheep. Considering the above studies, the evaluation of different allelic forms in major genes such as FSHβ is necessary. The aim of this study was the identification of allelic forms in exon 1of FSHβ gene in Baluchi and Nainie sheep breeds in Iran.

Materials and Methods

Animal and DNA extraction

Blood samples were randomly collected from 109 Naeinian (n= 70) and Baluchi (n= 39) sheep breed. Genomic DNA was isolated from blood samples using modified salting out method. Quality and quantity of extracting DNA was measured by 1% agarose gel electrophoresis.

Amplification of FSH gene

After DNA extraction, a 220 bp fragment was amplified in exon 1 of this gene. The PCR mixture contained 1 µl of each primers (forward primer, 5′-CGTCCAGTTCTGCTTCCTTTT-3′ and reverse primer, 5′- GTGGGAATCAATGAAACCTGC -3′) (Zhang et al., 2011), 0.5 µl of dNTP and 2U of XT-Taq DNA polymerase (0.3 µl), 1.5 µl of DNA template, 2.5 µl of PCR 1X buffer, 1.5 µl $MgCl_2$ and 16.7 µl of dH_2O DEPC in a final volume of 25 µl. The PCR was performed as follows: denaturation 94°C for 30s, annealing 58°C for 30s and extension 72°C for 30s in 35 cycles. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel using ethidium bromide staining.

Single-Strand Conformational Polymorphism Analysis (SSCPA)

PCR products were resolved by SSCPA. For SSCPA a 4 µl aliquot of each amplicon was mixed with 8 µl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene-cyanol). After denaturation at 96°C for 6 min, samples were rapidly cooled on wet ice for 10 min to prevent reannealing of the single-stranded product and then loaded on 14% acrylamide: bisacrylamide (37.5:1) gels. Electrophoresis was performed using Vertical Slab Unit, VSS-100 (Akhtarian), at 4-5°C with 250 V for 18h in 1% TBE buffer. A constant temperature was essential for band sharpness and reproducibility of strand separation. Then the electrophoresis unit was coupled to a refrigerator at 4°C. DNA fragments were visualized by the silver staining (Byun et al., 2009).

Results

After DNA extraction from blood samples by using modified salting out method, all of the samples amplified by a specific pair primer. Amplification of exon 1 of FSH gene showed a 220 bp fragment on agarose gel both in Baluchi and Naeini breeds (Fig. 1).

Genotyping by PCR-SSCP

The results of this study showed eight bands (A, B, C, D, E, F, G and H) for the mentioned region (Fig. 2).

Descriptive statistics

Table1 shows descriptive statistics of obtained band patterns. In this study, the "A" band pattern in the total sample had a greatest frequency (0.2) and the "C" band pattern had the lowest frequency (0.06). In the Naeini breed, banding pattern of "G", with about 13 samples

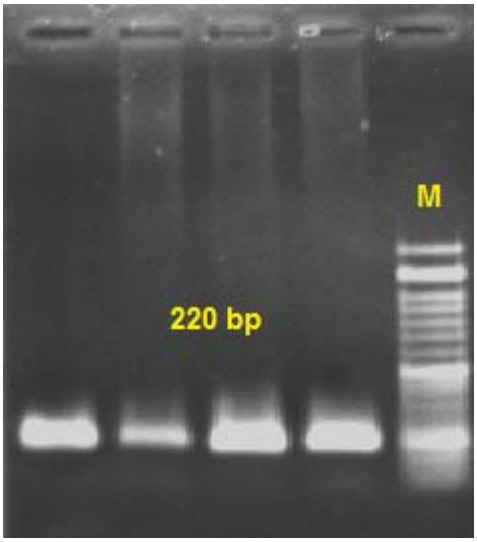

Fig. 1: The samples of amplification of exon 1 of FSHβ gene in present study

Table 1: The frequency of obtained binding patterns from PCR-SSCP analysis

The frequency of observed genotypes				Pattern
Total	Total	Baluchi Breed	Naeinian Breed	
0.2018	22	11	11	A
0.1284	14	2	12	B
0.0642	7	4	3	C
0.1376	15	5	10	D
0.1376	15	6	9	E
0.1284	14	9	5	F
0.0734	14	1	13	G
0.1284	8	1	7	H
1	109	39	70	Total

had the most frequency and in Baloochi breed, "A" band pattern with 11 samples showed the highest frequency in corresponding breed.

Sequencing results

The sequencing results in present study were confirmed by the PCR-SSCP analysis except in one case. The obtained sequencing results from C and G patterns was same, so the results of direct sequencing revealed seven unique sequences (Fig. 3). It is noticed that observed polymorphisms in this region led to no change in amino acid sequence.

In general, comparing the nucleotide sequences with the sequence of ovine FSHβ subunit published on GeneBank (accession number S64745.1), a nucleotide insertion in non-coding region of FSH (110 A-T→ACT) was detected. Moreover, sequencing results revealed three other mutations. Resulting eight different patterns for exon 1 of FSHβ gene among which two mutations (silent polymorphism) in positions 30 and 90 accrued in coding regions of exon 1 of FSHβ gene (Fig. 3). The deduced amino acids of the nucleotide sequences of different patterns were identical, so these mutations were silent.

Discussion

Reproduction is a quantitative trait with polygenic heredity. In other word, the reproductive activities in sheep are affected by many genes. Recently, numerous studies reported the reproductive traits which are influenced by major gene too.

For example, in Australia and New Zealand, a new breed of merinos' sheep have identified that have high litter size and theirs reproductive rate are controlled by major genes. Therefore, nowadays, it can be concluded that reproductive trait are controlled by both of major and minor genes. The ovulation rate in mammalian are determined by a complex exchange of hormonal signals between pituitary and ovary and also by a hormonal exchange inside of ovary follicles between oocyte and somatic cells (McNatty et al., 2005). Due to existence of many variation in ovulation rate in sheep different breed in world, sheep is as a good model to evaluate of growth and genetic control of ovulation rate (Montgomery et al., 2001). A mutation in nucleotide 680 of FSH receptor can cause a change in amino acid (Asparagine to Serine) which causes decrease of FSH activity. Also, some of mutation in FSH receptor lead to a delay in initial and final stages of follicle growth (Touraine et al., 1999). FSHα in mammalian is common and in general, biologic effect of FSH hormone is depend of specific activity of FSHβ. Beta subunit of FSH gene has three exons and two introns (Cui et al., 2009). The present study indicates that Iranian breeds (Baluchi and Naeini) have diversity in exon1 FSHβ gene. Li et al. (2010) did not observe any polymorphism on exon 10 of FSHR in Chinese Haimen goats using PCR-SSCP. Similar results were also observed in Xinong Saanen, Guanzhong and Boer goat breeds (Yuntao & Binyun, 2007). A study demonstrated that a single nucleotide polymorphism in the upstream p(-278 G to A) of FSH receptor gene led to create three types of genotype including CC, CD and DD in Chinese Holstein cows. Cows with CC genotype had a significant increase in the total number of ova (TNO) ($P<0.01$), and produced more transferable embryos (NTE) than those of the CD and DD genotypes ($P<0.01$) (Yang et al., 2010). The levels of gene expression of FSHR, FSHβ and BMP15 in Yeanling goats was less than Boer goats, while the gene expression levels of ESR2 and BMPR1B genes was more than Boer goats. Evaluation of polymorphism in exon 2 of follicle-stimulating hormone beta subunit showed three genotypes (EF, EE and FF) and also, showed a significant association with birth rates in the Boer and Saanen goat breeds (An et al., 2009). Lin et

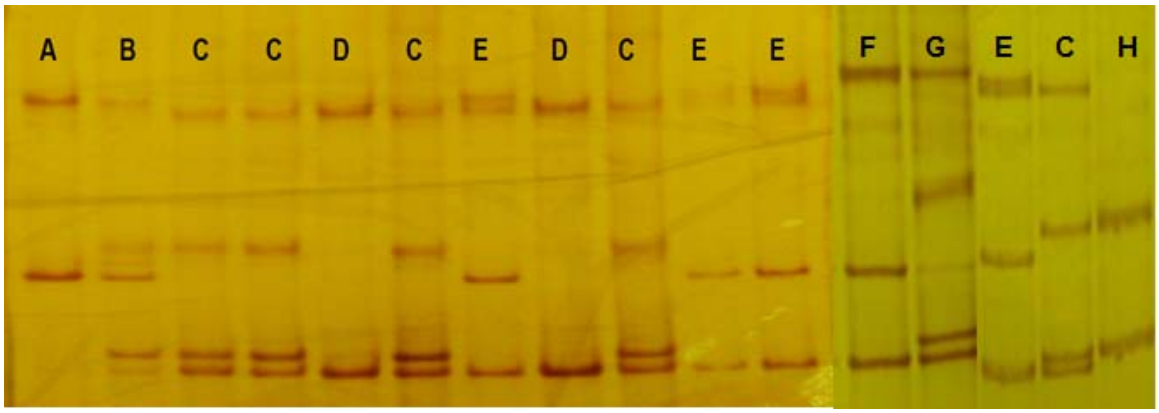

Fig. 2: Genotyping of amplified fragments of exon 1 using PCR-SSCP technique

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                  10        20        30        40        50        60        70
            ....|....|....|....|....|....|....|....|....|....|....|....|....|
S64745.1    AGCTGCGAGCTGACCAACATCACCATCACCGTGGAGAAAGAGGAATGTAGCTTCTGCATAAGCATCAACA
10          ............................G.................................................
20          ...........-................N.................................................
45          ............................N.................................................
54          ...........-.................................................................
82          .............................................................................
90          ............................G.................................................
123         ............................G.................................................

                  80        90        100       110       120       130       140
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
S64745.1    CCACGTGGTGTGCAGGCTATTGCTACACCCGGGTAGGCA-TTTGCTTTGCTGGAAGTGAGGGTGCTGAGG
10          ................C.....................C.........C.....................
20          ................N.....................C.............................
45          ..............................................C.............................
54          ..............................................C.............................
82          ................N.....................C.........N.....................
90          ................N.....................C.........N.....................
123         ................N.....................C.............................

                        150       160       170
                  ....|....|....|....|....|....|...
S64745.1          GTCTAGACAAGGCAGGTTTCATTGATTCCCACT
10                ................................-
20                ................................-
45                ................................-
54                ................................-
82                ................................-
90                ................................-
123               ................................-
```

Fig. 3: Multiple alignment of obtained nucleotide sequences from direct sequencing

al. (2006) reported polymorphism associated with fertility in FSHβ gene. The results showed CC, AA and AC genotypes in Boer goat and AB, AA and AC genotypes in Jining Grey goat respectively. AA genotype had the highest birth rate in both of two breeds (Lin et al., 2006). Liang et al. (2006) used nine primer pair for evaluation of polymorphism by PCR-SSCP in FSHβ gene. For this purpose, 5′- flanking region, exon 1 and 2 was evaluated and the results showed three genotypes AA, AB and AC in Jining and Cashmere goat, three genotype CC, AA and AC in Boer breed and six genotypes CC, AA, BB, AB, BC, and AC in Angora goat breed (Liang et al., 2006). Also Ren et al. (2007) studied the 5′-UTR polymorphism of FSHβ gene in Maiwa yak and Jiulong yak and

observed three different genotypes and reported that this gene can be used as a major gene which either control the reproductive performance or have close association with other major genes (Ren et al., 2007). In another study, polymorphism study in exon 3 of FSHβ genes showed relationship with birth rates, birth weight, length of pregnancy period and litter size in Mato and Boer goat breed and a new mutation in exon 3 of this gene was identified (Zhang et al., 2011). Recently, using RT-PCR technique analyzed the mRNA expression levels of FSHβ gene and its relationship with the birth rate in Magnolia goat breed and researchers reported that the complete cDNA of FSHβ gene consisted of 580 base pairs which code a protein with 129 amino acid (Yi et al., 2011). The alignment analysis of FSHβ gene sequence indicated a similarity of 99.2 and 98.7 percent between Magnolia goats and Black Yeanling and Magnolia and Boer goat breed respectively. in addition, mRNA expression levels of FSHβ in three breeds was associated with the birth rate and the correlation coefficient between the two Magnolia goats, Black Yeanling, Magnolia and Boer was 0.973 and 0.954, respectively (Yi et al., 2011). Sheep with AC genotype in comparison to the AA genotype of FSHβ gene in Baluchi sheep breed had a significant differences in birth rates trait (Nazifi et al., 2015).

It is concluded that Iranian Baluchi and Naeini sheep breeds have a chance to manipulate exon1 FSHβ gene for enhanced reproductive traits.

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