



Association of Leptin Gene Polymorphism with Growth Rate in Lohi Sheep

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ABSTRACT

Leptin hormone, encoded by LEP gene, has significant importance in regulating various functions like growth, puberty, reproduction and milk production in both animals and humans. The sequence variations have been studied, reported and associated with many growth traits like weaning weight, six month body weight and nine-month body weight etc. in cattle, buffalo, goat and sheep worldwide. LEP gene polymorphism and associations have not been extensively reported in Lohi sheep of Pakistan, so this study aimed to find any variations in gene sequence and possible association with growth rate to explore meat potential of this breed. A total of 18 Lohi animals were selected from the flock. After the DNA extraction from blood samples, a 1486 bp fragment of exon three was amplified and sequenced. Sequence analysis revealed single nucleotide polymorphism (T>A at position 483) and a heterozygous condition (T>W at position 483). Generalized linear model was used to minimize the environmental effects. The variation *i.e.* "W" type heterozygous condition showed a higher average daily weight gain. Sequence analysis of LEP gene confirmed the presence of genetic variability among Lohi animals; this variation might be associated with other economic traits for future breeding programs and marker assisted selection.

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Authors' Contribution

AAH and AA conducted the lab work. MZF assisted in data collection. KJ and MEB did the statistical analysis. TH and MA did the molecular analysis. Afzal A and NA helped in drafting of the manuscript.

Key words

Lohi sheep, DNA, Polymorphism, Exon, Leptin.

INTRODUCTION

In a developing country like Pakistan, small ruminants (sheep, goat) play an important role in sustainable livestock production. From the latest economic survey of Pakistan, it is evident that they have contributed 17.26% to the national meat production during 2016-17. The per capita consumption of meat in the country is 20.3 Kg and of mutton is 3.8 Kg, which is very low as compared to developed countries. Sheep is one of the important livestock assets in Pakistan having about 15.73% share (30.1 million) in total livestock population (191.3 million) of the country (Anonymous, 2017). Sheep farming adds up a great part to the agricultural economy particularly in the arid, semi-arid and hilly regions where crop production and dairy farming is not feasible. There are about 30 domesticated sheep breeds found in the country that produce meat, milk, and wool (Ali *et al.*, 2011). It is assumed that there are certain sheep breeds which have a higher potential for mutton and wool production (Babar, 1994), therefore exploring their genetic potential might be helpful to fulfill

the increasing meat demands in the country. Lohi is an important thin tail dual purpose sheep breed which is found in the central districts of province Punjab, Pakistan (GoP, 1996; Fiaz *et al.*, 2017). According to Babar *et al.* (2004), body features of this breed make it favorable for mutton production.

It is evident that hormones are involved in many processes of the body like growth, development, and reproduction (Neave, 2008), leptin is one of them. It is encoded by LEP/ob gene, which is located on the 5th chromosome in ovine. Javanmard *et al.* (2008) described that it has three exons and two introns, but only exon two and exon three are translated into protein. Among the meat production traits, average daily weight gain is the most important trait of consideration. Globally, LEP gene polymorphism in sheep has been discussed by many scientists like Shojaei *et al.* (2011), Hajihosseini *et al.* (2012), Bahrami *et al.* (2013), Mahmoud *et al.* (2014), Qureshi *et al.* (2015), Meena *et al.* (2016) and Quirino *et al.* (2016). It can be said that the variations in the production performance of animals might be due to the effect of genetics, environment and/or due to the interactions of both. Many researchers worked on large ruminants *i.e.* cattle and buffalo and most of the work was on the association of leptin gene with carcass traits, though

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some literature regarding growth and milk production traits is also available (da Silva *et al.*, 2012; Woronuk *et al.*, 2012; Mancini *et al.*, 2013). At present, inadequate information about LEP gene variants and their association with average daily weight gain is available in ovine population of Pakistan. Considering this deficiency present study is intended to find single nucleotide polymorphism (SNP) or variation in the LEP gene and its relationship with average daily weight gain in Lohi sheep.

MATERIALS AND METHODS

Animal resources and DNA extraction

Data regarding pedigree and body weights were collected from the flock of Lohi animals maintained at Small Ruminants Training and Research Center, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, Punjab, Pakistan. The animals were already vaccinated against fatal diseases and drenched anthelmintic against gastrointestinal parasites. They were fed green fodder, concentrates and *ad lib* water following the routine farm practices; and were housed in sheds according to weather conditions. Average daily weight gain (ADG) was calculated from birth to three months of age. After the statistical analysis (generalized linear model), a total of 18 animals were randomly selected from the flock for blood sampling. Genomic DNA was extracted from whole blood samples (5 ml each) using method used by Babar *et al.* (2014).

Table I.- Primers for exon three of ovine leptin gene used on Lohi sheep samples.

Primer name	Sequence 5'-3'	Product size	Temp. (°C)
Exon 3.1F	AGAAGTAAGGGTCCAGGAAG	627 bp	53.4
Exon 3.1R	CTGAGAGGAGCAAGAGAGAA		53.3
Exon 3.2F	CCTGGAAGCCTCCCTCTACT	364 bp	58.0
Exon 3.2R	AGGGAGGAAGACTGCTGTGA		57.9
Exon 3.3F	CTGGGATTTTCACAGCAG	438 bp	51.0
Exon 3.3R	GGTCCTTCGAGATCCATT		51.2
Exon 3.4F	AGGGCTCTCAAGTTTGTTT	533 bp	54.4
Exon 3.4R	GCATGCAAATCCCAAAG		55.9

Primer designing and PCR amplification

Primers were designed by Primer3Plus software using Gene ID: 443534 available at NCBI gene database. A fragment of 1486bp covering entire exon three was amplified using 4 sets of primers (Table I). Touchdown PCR was carried out using Thermal Cycler (iCycler BioRad, USA) with initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec,

annealing 58-48°C for 45 sec and extension at 72°C for 45 sec. Final extension was given at 72°C for 10 min and storage at 4°C.

Sequencing analysis

The amplicons were purified using TIANquick Midi Purification Kit (Tiagen Biotech Beijing Co., Ltd.) and sent for Sanger's sequencing to 1st BASE Laboratories, Singapore. Molecular analysis was carried out using Codoncode Aligner ver. 5.0.2 (García-Varela *et al.* 2016) and MEGA ver. 6.06 (Tamura *et al.* 2013). Peaks were read out, compared and variations were marked.

Age correction and elimination of environmental effects

Certain correction factors were calculated to minimize the environmental effects (Babar, 1994). The following model was used;

$$Y_{hijklm} = \mu + Z_h + S_i + X_j + T_k + A_l + G_m + \epsilon_{hijklm}$$

Where, h, 1, 2 (number of years = 2); i, 1, 2 (number of seasons = 2); j, 1, 2 (sex of lamb born = 2); k, 1, 2 (number of birth type = 2); l, 1, 2, 3 (age groups of dam = 3); m, 1, 2, 3 (groups of individuals on the basis of SNP = 3); Y_{hijklm} , ADG of nth lamb of mth genotype, jth sex, kth type of birth and lth age of dam and born during ith season in hth year; μ , overall population mean; Z_h , effect of hth year; S_i , effect of ith season; X_j , effect of jth sex of lamb born; T_k , effect of kth birth type; A_l , effect of lth age group of dam; G_m , effect of mth genotype; ϵ_{hijklm} , random error associated with ADG of nth lamb of jth sex, kth type of birth and lth age of dam, born during ith season in hth year and of mth genotype. It was additionally presumed that ϵ_{hijklm} was generally and autonomously distributed with mean 0 and variance σ^2 .

Association study

Analysis of variance technique was used for the estimation of environmental effects. After the molecular analysis, the animals were divided into three groups on the basis of observed SNPs. Student-Newman-Test was used in General Linear Model (GLM) of SAS (9.1) to calculate statistical differences among means of different groups.

RESULTS AND DISCUSSION

Two years' pedigree and performance data of ewes were used in the present study. Analysis of variance was performed by GLM of SAS ver. 9.1 software for the estimation of environmental effects. Statistical analysis revealed that birth weight, average daily weight gain, and coefficient of variation were 3.71 ± 0.09 kg, 117.90 ± 4.14 g and 24.22 and 28.02%, respectively on an average. Statistical analysis for the influence of environmental effects on corrected ADG showed significant association ($P > 0.01$) of the type of birth, season of birth and sex

of lamb with average daily weight gain as shown in Table II. The exon three of LEP gene in Lohi sheep was amplified using four sets of primers as shown in Table I. The sequence analysis revealed single nucleotide variant (A) and heterozygous peak (W) at position 483; where W represents overlapping of two peaks A and T (Fig. 1). On the basis of the type of SNP, Lohi individuals were divided into three groups. Association of SNP with average daily weight gain revealed statistically non-significant ($P>0.05$) association as represented in Table III. Animals having SNP “A” at 483 position showed a highest average daily weight gain of 172.73 ± 18.10 g followed by 135.00 ± 18.19 g with “W” type of SNP, while wild-type animals had the lowest average daily gain 115.60 ± 9.57 g. This work is one of the initial studies on polymorphism in LEP gene in Lohi sheep of Pakistan. Single nucleotide polymorphisms (A and W) in exon three of LEP gene were observed in Lohi animals used in current study. For the association study, the animals were grouped on the basis of observed SNPs. Data of these groups were analyzed and the results obtained were compared to find out any association (Table III). Results exhibited statistically non-significant differences ($P>0.05$) among ADG of individuals having “T” and “A” type mutations. Comparatively higher average daily gain (172.73 ± 18.10 g) was observed in individuals having heterozygous “W” type mutation followed by “A” (135.00 ± 18.19 g) and “T” (115.60 ± 9.57 g) type. On the basis of current analysis, the “W” type SNP can be considered as desirable for increased daily weight gain.

Table II.- Analysis of variance for evaluation of environmental effects.

SOV	DF	SS	MS	F Ratio	P Value
YOB	1	534.09439	534.09439	0.49	0.4860 ^{NS}
SOB	1	35144.61052	35144.61052	32.21	<0.0001*
Sex	1	14832.35958	14832.35958	13.60	0.0004*
TOB	1	7971.88755	7971.88755	7.31	0.0082*
AOD	2	930.91957	465.45979	0.43	0.6540 ^{NS}
Error	89	97098.5995	1090.9955		
Total	95	156512.4711			

*Highly significant ($P<0.01$), ^{NS} Non-significant ($P>0.05$); SOV, source of variation; DF, degree of freedom; SS, sum of squares; MS, mean squares; F ratio, Fisher ratio; P value, probability value; YOB, year of birth; SOB, season of birth; TOB, type of birth; AOD, age of dam.

Table III.- SNP and average daily gain in Lohi groups.

Group	No. of animals	SNP	Site	Mean (g) $\pm$ SE
1	7	W	483	172.73 ± 18.10
2	5	A	483	135.00 ± 18.19
3	6	T/wild	483	115.60 ± 9.57

SE, standard error.

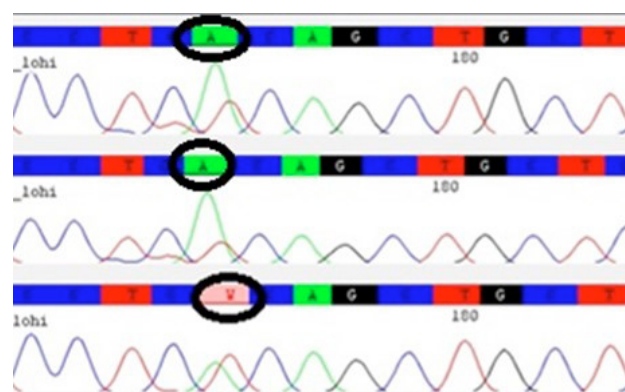


Fig. 1. SNP and heterozygous peak in exon three of LEP gene in Lohi sheep.

Lambs born in spring might be heavier at birth than lambs born in autumn because of the convenience of good quality fodder for the ewes during late winter and spring than the summer season (Babar, 1994). Lambs born as single had enhanced prospects in their mothers' uterus than twins or triplets. Male lambs usually halt somewhat longer in the uterus than female lambs, so heavier at birth (Babar, 1994). Many scientists found polymorphism in ovine leptin gene in their studies. Zhou *et al.* (2009) found four SNPs (C/T, G/A, G/A, G/T at positions 107, 271, 271 and 387, respectively) in exon three of leptin gene while working on Romney, Merino, Coopworth, Corriedale, Poll Dorset and Suffolk sheep breeds. Reicher *et al.* (2010) also found variations (A/G, A/C, AA/TC, G/A, G/T, and G/A at nucleotide no. 225, 228, 232-3, 312, 367 and 413, respectively) in exon three of leptin gene while working on Assaf breed. SNP patterns (L_1 , L_2 , L_3) found in LEP gene in sheep were significantly associated with additive estimated breeding values for weight at 90 days *i.e.* weaning weight (Tahmoorepur *et al.*, 2010). Shojaei *et al.* (2011) reported polymorphism and significant relationship with growth traits (SSCP patterns *i.e.* A/A, C/C, A/B, A/C, A/B/C, A/B/E, A/B/D/E, A/B/C/F, A/C/F and A/B/F) in exon three of leptin gene in Kermani sheep breed. An association between genotypes was found with weights at different ages of life in Makoei sheep (Hajhosseinlo *et al.*, 2012). Jonas *et al.* (2016) reported the relationship of observed SNPs in leptin (LEP) and leptin receptor (LEPR) genes with production traits and circulating leptin concentration in Awassi-Merino crossbred population. Meena *et al.* (2016) reported three SNPs in exon three of LEP gene in Malpura sheep breed using PCR-RFLP technique. The T387G locus was found polymorphic while A316C and A271G loci were monomorphic. Quirino *et al.* (2016) investigated variations in exon three of leptin gene and their association with carcass traits in Brazilian

sheep breeds *i.e.* Santa Inês (SI) and crossbreed (SI x Dorper). PCR-RFLP technique was used and among the three alleles of the gene, only one exhibited improved cold carcass weight. Three SNPs (G128T, C270T, and T166C) were observed by Wang *et al.* (2011) found novel SNPs (G128T, C270T, and T166C) in leptin receptor gene in goats, and associations between different genotypes and traits like prolificacy and birth weight of kids. Many haplotypes in exon two (DQ229928-30) and intron two (DQ229931-32) of LEP gene were reported by Singh *et al.* (2009) in different goat breeds. In cattle, Kulig and Kmiec (2009) found Sau3AI and A59V polymorphism while investigating the relationship of LEP gene polymorphism with some growth traits (body weight, average daily gain etc.). Results revealed that some A59V haplotypes had notable positive effects on body weight (at 210 d) and average daily gain (between 3-210 d). Tian *et al.* (2013) genotyped two SNPs (E2-169 T>C and E3-299 T>A) of LEP gene in steers and associated with the meat quality and carcass characters.

CONCLUSION

In summary, the molecular analysis of exon three of leptin gene of Lohi sheep showed polymorphism and trend of association with average daily weight gain. There is need to extend this work with more number of animals as well as on other sheep breeds to confirm the findings before declaring them as markers and using them for Marker Assisted Selection (MAS) in future breeding programs of Lohi.

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Statement of conflict of interest

The authors have no conflict of interest to declare.

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