

## Polymorphism of Leptin Gene and its Association with Milk Traits in Najdi Sheep

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The aims of this study were to characterize variations in *LEP* exon 3 region in Najdi sheep and assess their associations with milk and udder traits. A PCR amplicon of 471 bp was amplified and sequenced. Aligned DNA sequences showed two non-synonymous single nucleotide polymorphisms. At position G286T, three haplotypes were recovered; GG, TT and GT. For SNP G332A, only two homozygous haplotypes were recovered; GG and AA. The G-bearing genotypes (GG and GT) of G286T had an average of 27.2, 31.2, 28.9 and 21.2% higher values ( $P < 0.05$ ) for daily milk yield, total protein, total lactose and total milk solids than the T allele homozygotes at day 21 of lactation, respectively. At day 84 of lactation, the T allele homozygotes of G286T were significantly ( $P < 0.05$ ) associated with higher milk yield and milk constituents than the G-bearing genotypes (GG and GT); no differences ( $P > 0.05$ ) in milk and udder traits were detected between G allele homozygotes and GT heterozygotes of G286T. For the SNP G332A, genotypes were not significantly associated with daily milk yield, milk constituents and udder traits. It is suggested that different effects of Najdi genotypes on milk yield during suckling and milking periods may be due to differential expression of *LEP* gene in each period.

**Key words:** Leptin gene; Milk yield; Milk composition; Najdi sheep.

Sheep occupy a special niche in the Saudi Arabian agribusiness economy. The population of sheep is exceeding 7.2 million head, where Najdi sheep is considered the most popular breed among other indigenous breeds (Ayadi *et al.*, 2014). The Najdi breed is a fat-tailed sheep, known for their adaptability to the prevailing adverse environment of Saudi Arabia. It plays an eminent role in the rural economy, as lamb production is a main source of income in most of flocks. Najdi ewes are also used for milk production under the traditional bedouins rearing conditions. Recently, (Ayadi *et al.*, 2014) reported that this breed has noticeable potentials for milk production under intensive

production system. Additionally, there are emerging signs of consumer demand for dairy sheep products. Genetic variability assessment of the Najdi sheep is essential to develop a breeding program for selecting ewes that express superior milk traits and for the early selection of future breeders. Molecular genetic techniques for genetic improvement are currently available for direct genotyping individuals for specific genetic loci (Madeja *et al.*, 2004). Single nucleotide polymorphisms (SNPs) are the class of direct markers that can be used to locate functional mutations in coding loci and thus have the edge over other markers. Selection of suitable candidate gene, depending on the trait under study, and its pleiotropic effects are important to be investigated. *Leptin* (*LEP*) is one of these genes that can be

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used as a genetic marker to study its effect on traits concerning milk production in livestock (Liefers *et al.*, 2003).

Leptin, the expressed product of *LEP* gene, is a 16-kDa non-glycosylated protein synthesized and secreted predominantly by differentiated adipocyte cells. It is involved in the regulation of feed intake, energy balance, fertility, milk production as well as immune functions (Dubey *et al.*, 2012). The *LEP* gene is located on chromosome 4 in the ovine genome and consists of three exons and two introns. The gene structure, intron/exon boundaries and amino acid sequence, is highly conserved in mammalian species (Moravcikova *et al.*, 2012). Several SNPs in the coding region of the ovine *LEP* gene have been described (Zhou *et al.*, 2009) and were shown to be associated with food intake (Liefers *et al.*, 2002), growth traits (Hajihosseini *et al.*, 2012), and carcass and meat quality traits (Barzehkar *et al.*, 2009). In addition, polymorphisms in bovine *LEP* gene has been associated with milk performance traits (Kulig, 2005; Madeja *et al.*, 2004; Singh *et al.*, 2013). To date there has been no reported study demonstrating the association between ovine *LEP* gene polymorphism and milk traits. The aims of this study were to characterize potential variations in the exon 3 region of *LEP* gene in Najdi sheep and to analyze their associations with milk and udder morphology traits at days 21 (suckling period) and 84 (milking period) postpartum.

## MATERIALS AND METHODS

### Animals

The study was conducted on 31 Najdi ewes weighing 60-65 kg. The chosen ewes were three years of age and in their third lactation. All ewes lambed between January and March 2012 (26°C and 10% RH) in a semi-open sheds at the Experimental Animal Farm, King Saud University, Riyadh, Saudi Arabia. Ewes were managed similarly throughout the study, and fed 0.7 kg commercial pellets (DM basis; 14.53% CP, 1.16% EE, 24.91% NDF, 14.22% ADF, 0.54% Ca, 0.31% P, 7.46% ashes and 2.78 Mcal ME kg<sup>-1</sup> DM) in addition to *ad libitum* alfalfa hay. Fresh drinking water and mineralized salt blocks were freely available. At lambing, total lamb's weight produced by each ewe, litter size and gender were recorded. All lambs were

suckled their dams freely throughout the first 63 days of lactation (suckling period); thereafter, lambs were weaned and the ewes were milked once daily (milking period).

### Sampling and DNA extraction

From each ewe, at day 21 after lambing, milk yield potential in 24h-period was estimated; the estimation started after complete udder emptying with the aid of an i.m. injection of oxytocin (4 IU/ewe). To ensure complete and total milk letdown, two i.m. injections of oxytocin were given at 4-h interval. Milk yield potential in a 24h-period was calculated from milk yield in 4-h-period time six. After weaning, milk yield was recorded in the day 84 of lactation. The milking routine included, milking once daily with udder preparation and teats cleaning, hand stripping and teats dipping in 7% iodine solution immediately after milking. Measurements of udder depth (distance between the rear udder attachment and the base of teat), udder width (rear view of the longest horizontal line between the left and right sides of the udder) and udder circumference (maximum circumference perimeter) were recorded for all studied ewes at days 21 and 84 postpartum (Ayadi *et al.*, 2014). Assessment of udder health was performed by California Mastitis Test (CMT) using Bovivet CMT test kit (CMT Bovi-Vet, Kruse, Germany).

Milk samples (50 ml) were taken from each ewe, at days 21 and 84 after lambing for the determination of milk composition and DNA extraction. Protein, fat, lactose and total solids percentage were determined using a Milko Scan (Minor Type 78100, FOSS Electric, Denmark). For DNA extraction, 1 ml of each milk sample was centrifuged at 14000 rpm for 30 min at room temperature. The supernatant was carefully removed and the remaining cells pellet was washed twice with 1 ml of PBS (phosphate buffered saline) solution. Genomic DNA was extracted using the QIAGEN DNeasy blood and tissue DNA extraction kit (Hilden, Germany) following the manufacturer's instructions. The quantity and quality of DNA were checked by spectrophotometer (JenwayGenova Spectrophotometer Krackler Scientific Incorporation, USA) and the A<sub>260</sub>/A<sub>280</sub> ratios were between 1.7 and 1.9 indicating good quality DNA.

### Amplification of *LEP* exon 3 region

The amplification of *LEP* exon 3 region from sheep DNA preps was achieved by PCR using

*LEP*-up (5'-AGGAAGCACCTCTACGCTC-3') and *LEP*-dn (5'-CTTCAAGGCTTCAGCACC-3') primers. PCR reactions were carried out in a 25- $\mu$ l reaction volume containing 100 ng of template DNA, 1 $\times$  PCR KapaTaq Ready Mix (KAPA Biosystems, Boston, MA, USA), and 0.8  $\mu$ M of each 10  $\mu$ M primer. To reduce the possibility of cross contamination and variation in the amplification reactions, master mixes containing all PCR reagents except DNA template were performed. The amplification reactions were conducted using the Gene Amp PCR system 9700 (Applied Biosystems, Warrington, UK). The thermal profile consisted of an initial denaturation step for 2 min at 94°C, followed by 35 cycles of 94°C for 30s, 60°C for 40s and 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR products were assayed using 1% agarose gels and bands were detected by UV lamp after ethidium bromide staining through gel documentation system (Amersham Biosciences, Uppsala, Sweden).

#### DNA sequencing and sequence analysis

PCR products of *LEP* exon 3 region were cleaned and sequenced at the Advanced Genetic Technologies Center (<http://www.uky.edu/Centers/AGTC/>). The DNA sequences were edited and aligned using BioEdit software (Hall, 1999); (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>). The BioEdit software was also used to detect SNPs and indel mutations. The BLAST algorithm was used to search the NCBI GenBank database for homologous sequences (<http://www.ncbi.nlm.nih.gov/>).

#### Statistical analysis

Allele and genotype frequencies were determined by direct counting. A previous study using the same group of ewes (Ayadi *et al.*, 2014) did not detect any significant effects ( $P > 0.05$ ) for litter size and gender of born lambs during suckling and milking periods on milk yield, composition and udder measurements when the total weight of litters at birth was included in the statistical model. Therefore, the association analyses of the traits of interest were performed using the GLM of Statistical Analysis System (SAS version 9.2, SAS Inst. Inc., Cary, NC), according to the following model:

$$Y_{ijkl} = \mu + \beta W_{ijk} + G_j + D_k + e_{ijkl}$$

where  $Y_{ijkl}$  is the dependent trait,  $\mu$  is the overall mean of the trait,  $\beta W_{ijk}$  is the covariate effect of the total weight of litters at birth,  $G_j$  is the effect of the genotype for *LEP*,  $D_k$  is the effect of the day of milking, and  $e_{ijkl}$  is the random error component.

## RESULTS

A single PCR amplicon of 471 bp was amplified successfully from 28 DNA samples out of the 31 Najdi individuals used in the present study. The aligned 28 DNA sequences showed two non-synonymous single nucleotide polymorphisms (SNPs) at positions 286 (GTG/TTG-transversion), and 332 (CGG/CAG-transition mutation) of *LEP* exon 3 coding region. At the protein level, the 286 SNP resulted in replacing leucine with valine at 96 codon, whereas the 332 SNP resulted in replacing arginine with glutamine at the 111 codon. At position G286T, the heterozygote haplotype GT corresponds to the common type represented by 15 individuals, and homozygote haplotypes GG and TT represented by 7 and 6 individuals, respectively; the frequencies were 0.518 and 0.482 for the G and T alleles respectively. For the SNP G332A, the haplotype GG was the common type represented by 25 individuals, and the AA was the homozygote mutated; the G and A alleles had the frequencies of 0.893 and 0.107, respectively.

Statistical analysis revealed that one single nucleotide polymorphism in the coding *LEP* exon 3 region (G286T) showed significant ( $P < 0.05$ ) associations with milk and udder traits in Najdi ewes at day 21 postpartum (suckling period). The G-bearing genotypes (G allele homozygotes or GT heterozygotes of G286T) had an average of 27.2, 31.2, 28.9 and 21.2% higher values ( $P < 0.05$ ) for daily milk yield, total protein, total lactose and total milk solids than the T allele homozygotes of G286T, respectively (Table 1). The udder circumference was 11.6% smaller ( $P < 0.05$ ) in T allele homozygotes of G286T than the G-bearing genotypes (GG and GT). No differences in total milk fat and udder width and depth were detected between the observed three genotypes of G286T. For the SNP G332A, genotypes were not significantly associated with daily milk yield, milk constituents and udder traits.

After weaning the lambs, the results showed opposite trend (Table 2) for daily milk yield

and milk composition with different *LEP* exon 3 genotypes at day 84 postpartum (milking period). The T allele homozygotes of G286T were significantly ( $P<0.05$ ) associated with higher milk yield and milk constituents than the G-bearing genotypes (GG and GT); no differences ( $P>0.05$ ) in milk and udder traits were detected between G allele homozygotes and GT heterozygotes of G286T. For the SNP G332A, statistical analysis revealed that genotypes were not significantly associated with daily milk yield, constituents and udder morphology traits.

## DISCUSSION

The *LEP* exon 3 sequences in the Najdi ewes researched against other *LEP* sequences deposited in NCBI-GenBank database. Boucher *et al.*, (2006) detected four mutations in Dorset and Suffolk sheep, and two of these mutations were similar to the detected mutations in the present study. The representative *LEP* sequences from Dorset and Suffolk sheep that deposited in GenBank under accession no. AH014693 was identical to the G allele homozygotes genotype

**Table 1.** Least squares means of milk yield, composition and udder morphology traits in Najdi ewes at day 21 postpartum (suckling period), according to SNP genotype in *LEP* exon 3 gene

Trait	SNP G286T			SEM <sup>1</sup>	SNP G332A		
	GG	GT	TT		GG	AA	SEM <sup>1</sup>
Milk yield, g.d <sup>-1</sup>	2268 <sup>a</sup>	2105 <sup>a</sup>	1719 <sup>b</sup>	156.54	2062	2105	135.76
Milk composition, g.d <sup>-1</sup>							
Protein	91.1 <sup>a</sup>	84.5 <sup>a</sup>	66.9 <sup>b</sup>	12.68	75.6	80.2	10.68
Fat	113.8	101.3	100.1	14.62	102.1	113.1	9.41
Lactose	112.7 <sup>a</sup>	108.7 <sup>a</sup>	85.9 <sup>b</sup>	17.32	100.5	101.6	11.24
Total solids	327.2 <sup>a</sup>	298.1 <sup>a</sup>	257.9 <sup>b</sup>	44.71	288.3	311.5	30.98
Udder morphology, cm							
Circumference	48.2 <sup>a</sup>	46.9 <sup>a</sup>	42.1 <sup>b</sup>	3.25	45.4	47.9	2.11
Depth	19.4	20.2	19.5	2.18	19.9	19.7	1.15
Width	14.5	13.9	14.6	1.54	14.2	14.2	0.71

<sup>1</sup>Standard error of means

<sup>a, b</sup> Means in the same row within each SNP carrying different superscripts differ ( $P<0.05$ ).

**Table 2.** Least squares means of milk yield, composition and udder morphology traits in Najdi ewes at day 84 postpartum (milking period), according to SNP genotype in *LEP* exon 3 gene

Trait	SNP G286T			SEM <sup>1</sup>	SNP G332A		
	GG	GT	TT		GG	AA	SEM <sup>1</sup>
Milk yield, g.d <sup>-1</sup>	440b	355b	640a	56.63	437	439	46.32
Milk composition, g.d <sup>-1</sup>							
Protein	20.9b	18.7b	31.4a	3.58	22.1	20.7	1.98
Fat	23.9b	21.4b	36.1a	4.83	25.4	23.6	2.23
Lactose	17.8b	13.1b	23.2a	5.39	16.3	18.0	3.28
Total solids	64.1b	57.1b	93.4a	12.03	67.2	62.6	8.32
Udder morphology, cm							
Circumference	41.8	37.3	41.2	3.13	38.9	42.0	1.14
Depth	20.2	18.2	19.0	2.05	18.7	20.1	1.03
Width	13.4	12.2	11.5	1.63	12.2	13.5	0.29

<sup>1</sup>Standard error of means

<sup>a, b</sup> Means in the same row within each SNP carrying different superscripts differ ( $P<0.05$ )

recovered from the Najdi sheep breed. (Zhou *et al.*, 2009) used the same primer-pair and identified four SNPs in the Romney, Merino, Coopworth, Corriedale, Poll Dorset and Suffolk sheep breeds of New Zealand; only one of these SNPs was detected in this study (G286T). The SNP (A) at the positions 332 in Najdi ewes have been also found in Awassi breed (accession no. FR688118), whereas the SNP (T) at the position 286 was detected in Assaf breed (FR688115-FR688117); the four genotypes reported in Awassi and Assaf breeds are different from the Najdi sheep (Reicher *et al.*, 2011). Mahmoud *et al.*, (2014) detected three non-synonymous SNPs at positions 170 (GGG/AGG-transition), 286 (GTG/TTG-transversion) and 332 (CGG/CAG-transition) in Saudi indigenous Najdi and Naeimi sheep; two of these mutations (G286T and G332A) are also found in this study. In most of the studies, the DNA polymorphisms in the *LEP* exon 3 gene were associated with economical traits such as growth, milk production and reproduction.

This study investigates the potential of genetic analyses in the indigenous Najdi sheep. However, to our knowledge, this is the first study describing the association of milk production traits with polymorphisms in the exon 3 of the ovine *LEP* gene. Najdi ewes have been used for milk production under the traditional Bedouin's conditions, and recently under intensive production system. Unlike cows that usually wean the calves and milked immediately after birth, ewes are raised in a range of management options. In this study, Najdi ewes were not milked for the first 63 days post-partum until weaning, thereafter, ewes were milked once per day. The results showed that the genotypes GG and GT of G286T were associated ( $P < 0.05$ ) with an average of 467g more daily milk than the genotype TT at day 21 of suckling period, whereas, genotype TT of G286T at day 84 of milking period produced ( $P < 0.05$ ) an average of 242g more daily milk than the GG and GT genotypes. Divergent effects of the detected genotypes on milk yield during suckling and milking periods may be explained by the differential expression of the *LEP* gene in each period. It seems that the G-bearing genotypes are able to express their potentiality for higher milk production only under suckling condition. However, the difference in milk production before and after the weaning probably represent an environmental condition on

*LEP* gene expression. After weaning, it seems that the TT genotype of G286T is probably eligible to express its potentiality for lactation persistency. The persistency represents the rate of milk yield decreasing after the lactation peak; the slower decreasing is the more persistent. Many authors proved that more persistent lactation is desirable because it is related to better yield, animal health and reduction of feeding costs (Pulina *et al.*, 2007). Association between the *LEP* polymorphism and lactation persistency has not been investigated yet in sheep. Quantitative trait loci (QTLs) associated with lactation persistency and protein yield were mapped on chromosomes OAR3 and OAR20 in backcross between Awassi and Merino sheep (Singh *et al.*, 2007). Interestingly, none of these QTL is attributable to chromosome OAR4 where the *LEP* gene is known to be located. Therefore, in addition to leptin, other genes could be involved in the determination of phenotypic variation for traits such as persistency.

Several studies have been demonstrated the impact of bovine *LEP* gene polymorphisms on milk traits. In exon 3, two PCR-RFLP polymorphisms were described; namely, *HphI* (Haegeman *et al.*, 2000) and *NruI* (Lagonigro *et al.*, 2003). Positive associations were reported between *HphI* polymorphisms and the milk traits in Polish Holstein-Friesian (Madeja *et al.*, 2004), Sahiwal cows (Dandapat *et al.*, 2009), Jersey cattle (Kulig *et al.*, 2009) and buffalo (Mihaieu *et al.*, 2012). On the contrary, (Liefers *et al.*, 2002) found no significant differences in milk, protein and fat yields as well as in milk fat and protein percentage between Holstein-Friesian cows with different *HphI* genotypes. Singh *et al.*, (2013) reported that *NruI* and *HphI* polymorphisms in Sahiwal and Frieswal cattle of India were not significant predictors of the first lactation milk yield, peak yield and fat percentage; in *HphI* polymorphism, CT and TT genotypes have found to be higher protein and lactose percentage in comparison to CC genotypes. In addition, several RFLP polymorphisms were identified in bovine *LEP* exon 2; namely, *BspEI* (Haegeman *et al.*, 2000), *Kpn2I* and *BspI3I* (Buchanan *et al.*, 2002) and *Clal* (Madeja *et al.*, 2004). Buchanan *et al.*, (2003) genotyped Holstein cows by using restriction enzyme *Kpn2I* and found that individuals homozygous of the T allele produced 1.5 kg/day more milk compared to the



CC variant, without significantly affecting milk fat or protein percent over the entire lactation. Alashawkany *et al.*, (2008) found positive association between the *Bspl3I* polymorphism and milk yield of the 60- and 100-day in lactation, whereas no significant difference was observed between genotypes in milk yield of 305-day period. This effect was prominent in the 60-day period (1.7 kg/day), declining to 1.5 kg/day in the 100-day period. Liefers *et al.*, (2002) showed associations between the *Sau3AI* genotypes and daily milk yield and milk protein; these traits were significantly higher in the CT genotype compared with the CC homozygote ones. Liefers *et al.*, (2003) reported that *Sau3AI*-AB genotype produced 1.32 kg/day more milk and consumed 0.37 kg/day more food compared with the *Sau3AI*-AA genotype. However, (Kulig, 2005) found a higher daily milk, protein and fat yields for the TT genotype cows. On the other hand, no associations were found between the *Sau3AI* and *BspEI* polymorphisms and milk production in dairy cattle (Kulig, 2005; Madeja *et al.*, 2004). Such different results may be due to many factors such as breed differences, small number of cows, age of cow, lactation parity and stage, animal's health with special reference to udder, and interaction between the environmental factors and genetic components (Liefers *et al.*, 2003).

In conclusion, our results suggest that *LEP* is a potential candidate gene that can be used as a selectable marker in the breeding program for improving milk production traits in lactating Najdi ewes of Saudi Arabia. In selection for ewes that are capable to raise fast growing lambs with adequate amounts of milk for suckling, ewes with G-bearing genotypes of G286T could be preferred. On the other hand, identifying ewes carrying TT genotype may be helpful in selecting individuals for commercial milking under intensive production systems. However, given the limited number of observations, the resulted polymorphism effects could be false-positive associations. Therefore, further studies with larger numbers of Najdi ewes are required to confirm these results.

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