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Prolactin Receptor and Prolactin Genes Polymorphisms and their Associations with Milk Production Traits in Najdi Sheep

Mahmoud Ahmed Hossam

Zoology Department, College of Science, King Saud University,
P.O. Box 2455, Riyadh 1 1451, Saudi Arabia.

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Prolactin (PRL) hormone plays an important role in many biological processes in animals and humans, such as mammogenesis, lactogenesis and galactopoiesis. In dairy cattle, the *PRL gene* is a potential quantitative trait locus that is used to evaluate milk performance traits. We analyzed the sequence of prolactin (*PRL) and* prolactin receptor *(PRLR)* genes from 30 Saudi native sheep breed in order to investigate whether mutations in this gene can affect milk yield as well as composition. The genetic variability in the prolactin and prolactin receptor *(PRLR)* genes in Saudi indigenous sheep has been described. Three DNA fragments, a 156 bp fragment from PRL-exon 3 and two fragments represented in intron 1 (215bp) and intron 2 (176bp) of PRLR, were amplified and sequenced from Najdi sheep. The genetic effects of DNA polymorphisms on different traits related to milk production and composition were evaluated. Preliminary analysis showed no significant associations between PRL and PRLR genotypes and milk performance. There was only one haplotype (one version of these segments). Indigenous Najdi breed sheep was monomorphic at exon 3, intron 1 and intron 2. Further work is needed to investigate more individuals of Najdi sheep as well as other Saudi milk producing sheep breeds in order to understand the relationship between variability in PRL and PRLR and the milk yield and composition in these breeds.

Keywords: Prolactin gene, prolactin receptor gene, Najdi sheep, milk traits.

The population of sheep in Saudi Arabia is exceeding 7.2 million head (FAOSTAT, 2011), where Najdi sheep is considered the breed of choice. The Najdi or Nejdi is a breed of domestic sheep native to the Najd region of the Arabian Peninsula. Though it is primarily raised in Saudi Arabia, Nadji sheep are also present in Kuwait, Jordan, Oman, and Iraq.

The Najdi sheep has a distinctive appearance that has even been celebrated in Saudi "sheep beauty pageants" not unlike livestock shows and sales in the West. They are a very tall breed, averaging 76-86 centimeters (30-34 inches) in height at the withers. They have long, Roman nosed faces with drooping ears. They are generally black with white faces and white on the legs and tail. 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 have been 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

* To whom all correspondence should be addressed.
E-mail: ahmdhusam@hotmail.com

for selecting ewes that express superior milk traits and for early selection of future breeders. Molecular genetic techniques for genetic improvement are currently available for direct genotyping individuals for specific genetic loci (Agarwal *et al.*, 2008). Single nucleotide polymorphisms (SNPs) are the class of direct markers that locate the loci, coding for the functional mutation and thus have the edge over other markers.

The prolactin gene (*PRL*) encodes an essential hormone for initiation and maintenance of lactation, milk protein genes expression, osmoregulation, growth and development, reproduction and immune functions (Zukiewicz *et al.*, 2012). The ovine PRL consists of five exons separated by four introns (Dai *et al.*, 2007). Based on the important roles of prolactin in animal production, PRL gene was considered as a possible candidate gene for selecting productive traits. Significant associations were detected between PRL polymorphisms and reproduction (Dai *et al.*, 2007) and milk traits (Lu *et al.*, 2010).

Many candidate genes, such as prolactin (PRL) (Lu *et al.*, 2010; Lu *et al.*, 2011), prolactin receptor (PRLR) (Zhang *et al.*, 2008), growth hormone (Mullen *et al.*, 2010), insulin-like growth factor 1 (Mullen *et al.*, 2011), and insulin-like growth factor 2 (Mullen *et al.*, 2010), have been associated with dairy performance traits of cattle. Among these candidates, the *PRLR* gene is the most important one because of its crucial role in transmitting signals from lactogenic hormones to milk protein gene promoters (Berkowicz *et al.*, 2011). PRLR has a major role in mediating PRL action in the mammary gland, and thus the *PRLR* gene is considered to be responsible for milk yield (Mehmannavaz *et al.*, 2009). A recent study has revealed that five nucleotide variations (AJ966356: g.1218 T>A, g.1219 T>A, g.1220 T>G, g.1267 A>G, and g.1268 OT) in the exon 3 coding region of the *PRLR* gene were found and that the polymorphism was linked with milk production traits in dairy cattle (Zhang *et al.*, 2008). A single nucleotide polymorphism (SNP) (JX087440: c.1658 T>A) in exon 10 coding region of the *PRLR* gene was identified in the Saanen dairy goat, and this mutation resulted in a decrease in milk yield (Sun *et al.*, 2008). Polymorphisms within the Najdi sheep *in PRLR* gene are still unknown.

The aims of this study were to characterize potential variations in the *PRL* and *PRLR* genes using single nucleotide polymorphism analysis and to associate the possible effects of individual SNPs on the milk yield, compositions and selected udder morphological traits at two periods in Najdi sheep breed and compare the results with some of other populations in the world.

MATERIALS AND METHODS

Animals

The study was conducted on 30 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-1 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 a 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 striping 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. Protein, fat, lactose and total solids percentage were determined using a Milko Scan (Minor Type 78100, FOSS Electric, Denmark).

DNA was extracted from blood samples using the QIAgen DNeasy blood and tissue kit (Hilden, Germany) following the manufacturer's instructions. The exon 3, intron 1 and 2 of *PRL* and *PRLR* genes were amplified from Najdi sheep DNA samples by using three PCR primers (Table 1).

Polymerase chain reaction (PCR) amplifications were carried out in a 25 µl reaction volume containing 100 ng of template DNA and 2 µl of each 10 µM primer. To reduce the possibility of cross contamination and variation in the amplification reactions, master mixes containing all PCR reagents including the Kapa Taq polymerase enzyme (KAPA Biosystems, Boston, MA, USA) except DNA templates and primers were used. The amplification program was performed using the Gene Amp PCR system 9700 thermocycle (Applied Biosystems, Warrington, UK). The amplification protocol was initial denaturation step for 2 min at 94°C, followed by 35 cycles at 94°C for 30 s, 57 to 59°C annealing temperature (depending on the primer-pair) (Table 1) for 30 s and 72°C for 30 s. The final step of the amplification protocol was the extension step at 72°C for 5 min. All the reactions were carried out on 96 well PCR plates (Applied Biosystems, Warrington, UK).The quantity and quality of DNA were checked by spectrophotometer (Jenway Genova Spectrophotometer Krackler Scientific Incorporation, USA). The O.D. ratios were between 1.7 and 1.9 indicating high quality of DNA as indicated by (Sambrook *et al.*, 1989)

DNA sequencing and sequence analysis

PCR products of prolactin and prolactin receptor 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 *et al.*, 1999)); (Hall 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).

RESULTS AND DISCUSSION

Thirty individuals representing the indigenous Saudi sheep breed (Najdi) generated 156 bp PCR amplicons *of PRL exon* 3 region using *PRL-up* and *PRL-down* primers; 215 bp of *PRLR* intron 1 and 176 bp of *PRLR* intron 2 using *PRLR-up* and *PRLR-down* for both introns.

Sequencing of the PCR products from all individuals revealed identical sequences for the three regions. Some researchers used restriction enzymes to detect the mutation in 156 bp region of bovine using *Rsa*1 restriction endonucleases. In the present study the recognition site of the *Rsa*1 is probably absent; therefore, there was no mutation in Najdi sheep in this site.

Staiger *et al*. (2010) stated that *PRL* genotype had a significant effect on milk yield. Ewes carrying one allele produced 110.6 g more milk per day than ewes with the A alleles. The increase in production associated with the *A* allele (110 g of milk/d) has economic relevance because it represents about 7% of the average test-day milk in the study population.

Only a few studies have been conducted to investigate the association between prolactin gene polymorphism and milk traits in sheep (Ramos *et al.*, 2009; Staiger *et al.*, 2010). There is no

literature data at the best of our knowledge on associations between *PRL* and *PRLR* genotypes and milk composition, milk yield in sheep populations.

Ozmen *et al*. 2011 determined the polymorphism in the prolactin receptor *PRLR* gene in Chios, White Karaman and Awassi, the native sheep breeds in Turkey. Two primer pairs were designed for polymerase chain reaction (PCR) amplification within intron 1 (These investigators had used 1 primer pairs for intron 1 (391bp) of the ovine *PRLR* gene amplification.

Ozmen *et al*. 2011 used two primer pair (391bp) for intron 1 *PRLR* gene amplification and exon 10 of the *PRLR* gene in sheep. A total of 160 amplicons (99 for intron 1 and 61 for exon 10) were subjected to DNA sequence analysis. For intron 1, 6 different haplotypes were determined. For exon 10, 7 different haplotypes were obtained. It was noted in particular that White Karaman and Awassi were similar to each other in both PRLR exon 10 and intron 1 haplotypes, whereas the Chios breed had a different variation.

Our results are in line with (Ozmen *et al*., 2011). The three haplotypes (HM437210, HM437211, and HM437214) were identical with Najdi sheep in intron 1, and also one of the sequences (AF042358) was identical to that of intron 1.

The most common haplotypes were haplotype 2 (HM437210) for White Karaman and haplotype 4 (HM437212) for Awassi. However, haplotype 6 (HM437214) was the most common for the Chios breed. They determined that the most common allele for the chios breed was the same in Najdi sheep breed in intron 1.

Intron 2 in our study is identical with haplotype 5 (HM437213) and is different in reference of accession no.(AF042358) in one SNP)C allele(and in Najdi sheep was)T allele(in same position.

Chu *et al*. 2009 had detected ovine *PRLR* gene polymorphism by PCR-SSCP. Three primer pairs were designed for PCR amplification within intron 1 and exon 10 of the *PRLR* gene in sheep. Three genotypes (AA, AB and BB) were detected by the three primer pairs.

The prolactin receptor *PRLR* gene was studied as a candidate gene (Ran *et al.*, 2011) for the prolificacy of Jining Grey goats. Polymorphisms in intron 1 and intron 2 of *PRLR* gene were detected in high prolificacy (Jining Grey) and low prolificacy (Boer, Wendeng dairy, Liaoning Cashmere and Beijing) native goats using PCR-SSCP. For intron 1, five genotypes (AA, AH, AK, HH and HK) were identified in Jining Grey goats, and two (AA and AK) in the other four breeds.

Li *et al*. 2010 identified the relationship between genetic polymorphisms at three reproduction associated loci and litter size were explored in Chinese Haimen goat. The results showed that intron 1 of prolactin receptor *PRLR* gene had two genotypes and three genotypes were also found in intron *2* of the same gene.

Alfonso *et al*. 2012 analyzed the polymorphism of prolactin gene as well as its relationship with milk production in American Swiss cattle in six herds using *Rsa*l restriction endonucleases. Showing a 156 bp fragment located in exon 3. They concluded that the structure of the studied populations showed similarities with regard to productive characteristics, as a consequence of the use of genetic material from the same origin.

Dybus *et al*. 2005 analyzed the associations between polymorphism localized in the third exon of the prolactin gene *(PRL-Rsa1*) and milk production traits of Black-and-White and Jersey cattle. They found no associations between *PRL* gene and milk production traits for Black-and -White cattle.

Table 1. Primers of PRL and PRLR gene designed for PCR analysis

Primer	bp	Forward	Reverse	Tm	Reference
PRLRIntron1	215	CATCTGCTGG AGGTAAGTGC	TTCATTGCCT CTGACGCTT	59	Ran *et al*.,2004
PRLRIntron2	176	TGTCAGTAAG CGTCAGAGGGC	GGCTGGTGGA AGGTCACTCTT	59	Ran *et al*.,2004
PRLExon3	156	CGAGTCCTTAT GAGCTTGATTCTT	GCCTTCCAGAA GTCGTTTGTTTTC	57	Mitra *et al*.,1995

Mehmannavaz *et al*. 2009 found the effects of prolactin SNP on genetic trends and the difference between genetic trends produced by two the alleles were not significant for all studied traits in Iranian Holstein bulls.

Kaplan and Boztepe. 2010 determined the prolactin gene *(PRL-Rsal)* polymorphism within Indigenous Anatolian Water Buffalo breed and Brown Swiss cattle, the results showed monomorphic at exon 3 *(PRL-Rsal)* loci in Indigenous Anatolian Water Buffalo, prolactin gene mutation in exon 3 *Rsa*l digestion site is not observed in 45 Indigenous Anatolian Water Buffalo breed. This result is identical to the results have been reported by (Mitra *et al.*, 1995) These researchers who carried out the study about exon 3 *PRL-Rsa*l loci in Murrah, Nili Ravi and Egypt buffalo breed stated that they have observed mutations in Murrah and Nili Ravi buffalo breed, whereas they have not observed any mutation in Egypt buffalo breed.

Studies on the *PRLR* gene have concentrated more on porcine breeds (Kernerová *et al.*, 2009). A mutation was identified in the porcine *PRLR* gene (Vincent *et al.*, 1998). An *Alu1* PCR-RFLP polymorphism was identified in the porcine 457 bp-long fragment of the *PRLR* gene (Kernerová *et al*., 2009). A new *Hpa1* PCR-RFLP polymorphism was identified in the porcine *PRLR* gene (Putnova *et al.*, 2009).

CONCLUSION

Reports in nucleotide mutations in sheep of *PRL* and *PRLR* gene are not available at the best of our knowledge. To date, the relationship between the *PRL* and *PRLR* genotypes and milk performance of Najdi breed sheep has not been reported. In the present study, it appears that there is no relationship between polymorphism in either *PRL* or *PRLR* gene and milk yield and milk composition. Further genes must be targeted in order to find a correlation between milk traits and other genetic markers.

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