QUANTITATIVE GENES SEQUENCING IN KARADI EWES ASSOCIATED WITH MILK YIELD TRAITS

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Abstract. The objective of present study was to investigate sequencing of four genes (AlphaS1-casein, alphaS2-casein, beta lactoglobulin and Major histocompatibility complex) that related to milk traits in Karadi sheep. A total of 300 ewes from three flocks were studied. The results revealed which are include effects of lamb's sex, dam's age and month of lambing were not significant on the milk traits except the ewes flock has significant effect on daily milk yield. The Best Linear Unbiased Prediction value for all ewes concerning daily milk yield, protein and fat percentages ranged from -10.5293 to 10.7504, -2.0546 to 2.0097% and -1.7033 to 1.4067%, respectively. The DNA sequencing results show that there are differences between ewes with high and low milk production in AlphaS1-casein, alphaS2-casein, beta lactoglobulin and Major histocompatibility complex loci sequences these differences reflected by milk performance of ewes in two groups, ewes in high group produced 300% more daily milk than ewes in low group (355.8 vs. 102.8 g/day). In conclusion results showed that there are agreements between Best Linear Unbiased Prediction value with DNA sequencing results in the select best animal and the selection process with molecular technique can play a major positive and rapid role to improvement and increasing milk production in this breed of sheep.

Keywords: Karadi Sheep, BLUP, Daily Milk Yield, Fat%, Protein%, DNA Sequencing

Introduction

In Kurdistan, there are many native sheep breeds with different productive and reproductive performances. One of the most important of them is the Karadi sheep that reared mainly for milk, meat and wool production. Solid content of ewes milk is a higher than other farm animals (cow and goat), which means the sheep milk is particularly suited to produce cheese and yogurt (Bencini and Johnston, 1997; Gutiérrez-Gil et al., 2014). After weaning all milk is used for yogurt and cheese production, for that milk content are very important (Carta et al., 2009). The total number of sheep in Iraqi-KRG was 7,722,372 heads (Al-Alaq et al., 2011).

The research to locate the gene responsible for the prolificacy in this breed, using molecular genetic markers, may help its use commercially to improve other local breeds. The polymorphism of blood genetic markers gives some useful information in studying the relationships among breeds and their evolution. It can also be used for indirect selection if there were some relationships between these markers and some economically important quantitative traits (Anous et al., 2009). Molecular methods have also provided new markers for the study of genetic variation and evolutionary relationships of closely related populations (Visser et al., 2004; Lamartino et al., 2005;
Kumar et al., 2005). Improvement of livestock has focused on the selection of breeding individuals with superior phenotypes. With the development of increasingly advanced statistical methods that maximize selection for genetic gain, this simple approach has been extremely successful in increasing the quantity of agricultural output and productivity. However, information now available on the organization and functioning of the genome could be used in breeding programmers to improve a range of traits. While genetic markers for QTL that are linked to the trait gene could be used to choose animals for selective breeding programmers, the most effective markers are the functional mutations within the trait genes. Strategies to identify markers for traits and the application of these markers are described by reference to examples of loci that control a range of different traits (Williams, 2005).

Therefore, the objective of the present study is to assess the genetic structure within the Karadi sheep population at the DNA level in order to find molecular genetic markers which can differentiate between females with high and low production and help for the identification of the prolificacy gene in Karadi sheep in Iraqi-KRG.

Materials and methods

Animal and DNA isolated

Experimental done on nearly 300 Karadi ewes (2-5 years old) from three private flocks at different locations of Sulamania governorate Arbat District (Latitude, 35° 25’ 14”W, Longitude, 45° 03’ 36”W, elevation 681 m), Sharazoor District (Latitude, 35° 15’ 27”W, Longitude, 45° 42’ 21”W, elevation 614 m) and Mawat District (Latitude, 35° 52’ 34”W,45° 24’ 35”, W, elevation 858 m), Karadi ewes were studied for their milk production with percentage of protein and fat in ewes milk for one season (5 months), milking methods depend on ICARDA (1995). All necessary information was recorded for each ewe (age of dam, sex of lamb, month of lambing, daily milk production, fat%, and protein %). Protein% and fat% of milk were estimated from the milk sample monthly using milkoscan TM minor machine (P/N 6004 4208, Issue 1 GB, March 2010, FOSS Analytical, 69, Slangerupgade, DK 3400 Hillerod, Denmark).

Whole blood (5 ml) was collected from each ewe from jugular vein into 10 ml Vacutainer tubes containing EDTA for genetic studies. Genomic DNA was extracted from whole blood using Quick-DNATM Miniprep Kit (ZYMO RESEARCH CORP, USA). Following the extraction, the quality and quantity of the extracted DNA samples were assessed using a Nanodrop spectrophotometer 2000 (UK). Laboratory work was done in the postgraduate laboratory at the faculty of agricultural sciences, Sulaimani University, Sulaimani.

PCR amplification and genotyping

Four specific genes were used and the primer sequence and their PCR condition are shown in Table 1. PCR reaction was carried out for forward and reverse primers in 50 μL of total volume, containing 10 X PCR buffer (50 mM/L Kcl, 10 mM/L Tris-HCl (pH 8.0), 0.1% Triton X-100), X mM MgCl2, 0.2 mM of each dNTP, 10 pM/L of each primer, 50 ng ewe genomic DNA and 1U Taq DNA polymerase.

The PCR Thermal Cycler (TC9610 /TC9610-230, Applied Bio systems, USA) was done in a final reaction volume of 50 μL. A master mix for all samples for each gene was readied and 40 μL filled in every PCR tube. Ten μL of DNA sample was added to
each tube to make the last volume 50 μL to accomplish homogeneity of reagents and decrease the risk of contamination, control reaction was situated up without genomic DNA. A GoTaq® Green Master Mix (ADM7122 00000311719, Promega-USA) incorporates with 25 μL Taq DNA polymerase (25 Units/mL, dNTPs 200 μM, and MgCl2 1.5 mM), 4 μL primer (0.1-1 μM, forward and reverse), 5 μL (100 ng) of DNA template and 16 μL DNase free water.

Ewes genotyped were done by using the PCR and direct sequencing. To confirm results, thirty randomly chosen PCR samples of each groups (High, and low milk yield) were sequenced from both directions. Direct sequencing was performed by commercial services using 3100 ABI PRISM sequencer (Applied Bio-systems, USA). Sequences were obtained with the same primers used for PCR amplification.

Table 1. The sequences and information of primers used in this study

| Gene       | Primer sequences 5′ --------- 3′ | PCR conditions                                                                 | PCR product size | References       |
|------------|---------------------------------|-------------------------------------------------------------------------------|------------------|------------------|
| α-s1 casein | TTGGGTTCAGTGGTGAAGTCTGG         | Initial 5 min 95 °C 30 cycles, 95 °C for 30 s,60 °C for 30 s,72 °C for 30 s, final elongation 5 min at 72 °C | 452 bp           | Corral et al. (2010) |
| α-s2 casein | CTGAAGTTGCCCCAGAGGTA CATTGGAGAAGAAGCAGTGG | Initial 95 °C for 5 min 35 cycles, 95 °C for 30 s,55 °C for 30 s, 72 °C for 1 min, final 72 °C for 5 min | 225 bp           | Rozen and Skaletsky (2000) |
| β-LG       | TTGGGTTCAGTGGTGAAGTCTGG         | 33 cycles,95 °C for 1 min,66 °C for 1 min,72 °C for 1 min, final elongation 5 min at 72 °C | 452 bp           | Jurate et al. (2005) |
| MCH class II DRB | TCTCTGCAGCACATTTCTCCGG CTCGCCGCTGACGTAAC | 35 cycles,95 °C for 1 min,60 °C for 1 min,72 °C for 1 min, final elongation 5 min at 72 °C | 258 bp           | Ammer et al. (1992) |

Bioinformatics analysis

Sequences were analyzed using the Chromas version 2.6.5 Technelysium Pty Ltd. Sequence analysis and alignments were carried out using NCBI BLAST: Nucleotide sequence. The nucleotide sequences of the four tested genes in Karadi ewes were submitted to GenBank (NCBI, BankIt).

Statistical and genotypic analysis

The PROC GLM (General Linear Model) procedure (SAS, 2002) was used to analyze the data for daily milk production (g), fat and protein %. Fixed effects studies
were flock, age of dam, sex of lamb, and month of lambing were fitted in the following model:

\[ Y_{ijklm} = \mu + F_i + A_j + S_k + M_l + \varepsilon_{ijklm} \]  

(Eq.1)

where: \( Y_{ijklm} \) = milk yield, fat and protein\% of \( m^{th} \) ewe, of \( i^{th} \) ewes flock (\( Fi, i = 1,2 \) and3), of \( j^{th} \) age of ewes (\( Aj, j = 2,3,4 \) and 5 years), of \( k^{th} \) sex of lambs (\( Sk, k = 1, \) male and \( k = 2, \) female) and of \( l^{th} \) month of lambing (\( Ml, l = \) Nov., 2 = Dec., 3 = Jan., and 4 = Feb.), \( \mu \) = Population mean, \( \varepsilon_{ijklm} \) = random error. It was assumed to be normally and independently distributed with mean zero and variance \( \delta^2 e \).

For genetics evaluation of ewes (High and low milk production) for various performance traits, Best Linear Unbiased Prediction (BLUP) procedure described by (SAS, 2002) was applied. The model used for this purpose was the Mixed Model (Fixed + Random effects) of (SAS, 2002) software. The individuals of the Karadi breed were assembled in three groups; high (30 ewes), medium (240 ewes) and low (30 ewes) production, according to the BLUP value for DMY: where 10% of ewes for the 1st group (Top milk yield group), 80% for 2nd group and 10% for 3th group (low milk yield group). The above Equation 1 was used to analyze the difference among ewes group after add the groups effect to the equation.

**Results**

**Phenotypic results**

**Fixed effect**

Least square means of milk traits were \( 236 \pm 10 \) g/day, \( 5.33 \pm 0.62\% \) and \( 5.30 \pm 12.93\% \) for DMY, protein\% and fat\%, respectively (Table 2). The milk traits curve show that the higher milk yield recorded after one month of ewes lambing was arrived \( 339 \) g/ewe/day and lowest at end of lactation, while Fat \% curve increase linearly after lambing to end of lactation showed the reverse way with milk production, but the protein % look like milk curve along lactation stage (Fig. 1).

As in Figure 2 the ewes flock have significant effect on DMY and Fat %, ewes in 3rd flock yield more milk compared with other two flocks.

![Figure 1. Daily milk yield (g), fat and protein % in Karadi ewes](image-url)
Table 2. Least square means ± SE for daily milk yield (DMY), protein% and fat% in Karadi ewes

| Fixed effects       | DMY (kg)       | Protein %  | Fat %      |
|---------------------|----------------|------------|------------|
| Overall mean        | 0.236 ± 0.10   | 5.33 ± 0.62| 5.30 ± 12.93|
| Flock               |                |            |            |
| 1                   | 0.220 ± 0.01a  | 5.33 ± 0.06a| 4.23 ± 1.27a|
| 2                   | 0.211 ± 0.02a  | 5.15 ± 0.16a| 6.81 ± 3.48a|
| 3                   | 0.312 ± 0.01b  | 5.46 ± 0.10b| 4.37 ± 2.09b|
| Ewes age (year)     |                |            |            |
| 2.5                 | 0.261 ± 0.01a  | 5.37 ± 0.08a| 5.31 ± 1.83a|
| 3.5                 | 0.245 ± 0.01a  | 5.30 ± 0.08a| 6.07 ± 1.72a|
| 4.5                 | 0.241 ± 0.01a  | 5.20 ± 0.09a| 4.71 ± 1.97a|
| 5.5                 | 0.244 ± 0.01a  | 5.39 ± 0.12a| 4.45 ± 2.48a|
| Sex of lambs        |                |            |            |
| Male                | 0.251 ± 0.01a  | 5.42 ± 0.11a| 5.37 ± 2.36a|
| Female              | 0.245 ± 0.01a  | 5.21 ± 0.09a| 4.90 ± 2.02a|
| Month of lambing    |                |            |            |
| November            | 0.247 ± 0.01a  | 5.34 ± 0.06a| 5.26 ± 1.34a|
| December            | 0.264 ± 0.01a  | 5.20 ± 0.12a| 5.58 ± 2.60a|
| January             | 0.246 ± 0.01a  | 5.50 ± 0.12a| 4.98 ± 2.59a|
| February            | 0.234 ± 0.02a  | 5.22 ± 0.14a| 4.72 ± 2.95a|
| Stage of lactation (month) |        |            |            |
| 1                   | 0.321 ± 0.01a  | 5.33 ± 0.08a| 4.83 ± 1.83a|
| 2                   | 0.339 ± 0.01a  | 5.51 ± 0.08a| 4.90 ± 1.84a|
| 3                   | 0.281 ± 0.01b  | 5.32 ± 0.08a| 7.62 ± 1.85a|
| 4                   | 0.201 ± 0.01c  | 5.31 ± 0.08a| 4.96 ± 1.84a|
| 5                   | 0.100 ± 0.01d  | 4.94 ± 0.09b| 5.21 ± 2.10a|

Means in the same column for each factor with different letters are significantly (p ≤ 0.05) different

Figure 2. Effect ewes flock on daily milk yield (g) and fat %
The milk traits did not affected by age of ewes, but ewes with 3 years old product more milk compared with another ewes age (Fig. 3). This may attributed to the biological condition and physiological maturity of three years old ewes. As well as sex of lamb also did not have significant effect on all milk traits in this study (Fig. 4).

**Figure 3.** Effect age of ewes on daily milk yield (g) and fat %

![Graph showing effect of age on milk yield and fat percentage.](image)

**Figure 4.** Effect lamb sex on ewe's daily milk yield (g) and fat %

![Graph showing effect of sex on milk yield and fat percentage.](image)

*Genetic merit (BLUP) of milk traits*

Best Linear Unbiased Prediction (BLUP) is generally used to predict animal breeding values, given measurements on progeny, or to predict breeding values of animals with repeated records, or to predict breeding values of all animals in the
pedigree (Cameron, 1997). Best linear unbiased prediction is one of the current methods of choice for genetic evaluation of quantitative traits. BLUP values overall ewes for DMY, protein and fat% were ranged from -10.52 to 10.75, -2.054 kg to 2.009% and -1.703 to 1.406%, respectively (Table 3). According to the BLUP values for high and low groups the results show that there are significant deference’s between ewes with high and low milk production group for all traits under studies as in Figures 5–7.

**Figure 5.** Daily milk yield (g) curve in high and low ewes group

**Figure 6.** Protein % curve in high and low ewes group
In this study, the sequencing analyses for high and low production groups of ewes were done for all genes under study.

*AlphaS1-casein (CSN1S1) locus*

The results show there are difference between high and low group (Figs. 8 and 9) the high group milk production match 100% with NCBI (Sequence ID: JN560175.1) while the low group match 99% with the same NCBI reference which have mutation point at 4298 (T-G), this mutation changed the amino acid from cys (UGU) to trp (UGG).
AlphaS2-casein (CSN1S2)

Ewes with low production match 99% with NCBI (Sequence ID: FN601350.1) which have mutation point at 176 (A C) this mutation changed the amino acid from His (CAC) to Pro (CCC), while ewes with high milk production match to the same NCBI 100% (Figs. 10 and 11).
Figure 10. Sequence alignment of Karadi sheep (High milk production group) CSN1S2 with published sequences

Figure 11. Sequence alignment of Karadi sheep (low milk production group) CSN1S2 with published sequences

The beta lactoglobulin (β-LG) gene

Both groups was match 100% to the NCBI (Sequence ID: X12817.1) without differences between both groups (Figs. 12 and 13).

Major histocompatibility complex (MHC class II DRB)

Ewes in high group milk production match 98% with NCBI (Sequence ID: Z92728.1) for Major histocompatibility complex (MHC- DRB) which have two mutation point, the 1st one found at position 139 (G T) this mutation changed the amino
acid from arg (CGG) to leu (CUG) and the 2nd one at 244 (G -), this mutation changed the amino acid from ser (AUG) to met (AUG), while low group production match 99% to same NCBI sequence with one mutation point at 244 (G C), this mutation changed the amino acid from ser (AGU) to thr (ACU) (Figs. 14 and 15).

As in DNA sequencing results for CSN1S1, CSN1S2, β-LG and MHC-DRB loci there are difference between ewes with high and low milk production groups, this differences in DNA sequencing reflected on milk performance of ewes in two groups, ewes in high group produced DMY 355.8 g/day compared with 102.8 g/day in low group.

Figure 12. Sequence alignment of Karadi sheep (high milk production group) beta-lactoglobulin with published sequences

Figure 13. Sequence alignment of Karadi sheep (low milk production group) beta-lactoglobulin with published sequences
Discussion

Phenotypic results

The average daily milk yield observed in this study was below the range indicated earlier by several researchers for several sheep breeds (Oramari, 2009; Abd Allah et al., 2011). However, protein and fat percentage were similar to other studies reported earlier by several investigators in different breeds of sheep (Bendelja et al., 2009; Abd El-Fatah and Awad, 2014). Our results showed that flock has significant effect on DMY and fat%. Similar results were recorded by (Maarof et al., 1986; Sanna et al., 1998; Ruiz et al., 2000; Al-Barzinji, 2003, 2009; Raaof, 2005, 2006; Gardi, 2008; Al-Barzinji and Abdul-Rahman, 2012; Al-Barzinji and Al-Rawi, 2012) on Iraqi sheep breeds. According the effect of ewe age our result is in agreement with many research works (Al-Rawi et al., 1997; Mavrogenis, 1996; Fuertes et al., 1998; Macciotta et al., 2000;
Al-Mohammadi, 2002; Al-Barzinji and Hassan, 2005; Raaof, 2005, 2006; Gardi, 2008; Al-Barzinji, 2009; Al-Barzinji and Al-Rawi, 2012. This result is similar to that reported by (Al-Barzinji, 2009; Al-Barzinji and Abdul-Rahman, 2012; Al-Barzinji and Al-Rawi, 2012) in Hamdani sheep breed which showed that sex of lamb had no significant effect on all milk trait in studied in this study.

The BLUP results in present study are similar to reported by Al-Barzinji and Abdul-Rahman (2012) for DMY and fat % in Hamdani sheep breed in Iraq. The BLUP value ranged from -10.5293 in low milk yield group to 10.7504 in high milk yield group. The BLUP value ranged from -2.0546% in low protein yield group to 2.0097% in high yield group and the BLUP value ranged from -1.7033% in low fat yield group to 1.4067% in high yield group. These results show significant differences among ewes groups, these differences return to genotypic effect among ewes group. The breeder can use these wide ranges of ability to produce more milk among ewes and can make selection process to increase the allele frequency of quantitative loci to speed up the improvement of milk yield which have significant effect on lamb’s performance in next generation.

**Genotypic results**

The results of the study Mroczkowski et al. (2004) indicate the superiority of sheep with CC αs1–CN genotypes of the milk yield, fat and protein percentage. Similar results are reported by Chianese et al. (1996), who analysed Sarda, Comisana and Delle Lanqhe crosses and observed the milk production level to decrease in the following order: BC > CC > CD. Animals with the CC genotypes were characterized by a higher percentage of fat and casein in milk compared to both the CD and BC genotypes. Piwczynski et al. (2002) reported that in a population of Polish Merino × prolific sheep, ewes with BC αs1–CN genotypes were highly significantly better in milk production than animals with AC and CC genotypes. However, CC homozygotes were better in percentage of protein and solids than AC and BC heterozygotes. Ovine milk containing CSN1S1 genotype CC showed a higher protein and/or fat content than AC, CD, DD, or CX milk (Chianese et al., 1996; Pirisi et al., 1999; Mroczkowski et al., 2004; Wessels et al., 2004). Therefore, CSN1S1 CC milk had better renneting properties, and better cheese-making characteristics than CD and DD milk (Chianese et al., 1997; Pirisi et al., 1999).

An investigation was carried out to explain characterize the ovine alphaS2-casein (CSN1S2) by Picariello et al. (2009) of three Italy sheep breeds showed that B variant differs from the most common form A with two amino acid exchanges: Asp75 Tyr75 and Ile105 Val105.

Investigations in many countries have shown that β-lactoglobulin is polymorphic in various breeds of sheep. Three co-dominant alleles (A, B and C) have been reported in this species differing by one or more amino acid changes. The genetic variant A differs from variant B in the amino acid sequence at position 20 (Tyr20→His20) (Bell and Mckenzie, 1967; King, 1969; Kolde and Braunitzer, 1983; Ali et al., 1990), the rare variant C is a subtype of A with a single amino acid exchange at position 148 (Arg→Gln) as reported by (Erhardt, 1989).

Pietrolà et al. (2000) did not find any direct effect or linkage between milk yield and β-lactoglobulin genotype. Ramos et al. (2009) observed higher milk yield in AB heterozygotes in Merino and Serra da Estrela sheep. In addition, the Serra da Estrela AA ewes presented lower milk yield when compared with AB animals with no significant difference AB and BB genotypes Ramos et al. (2009). Kawecka and Radko...
(2011) found no associations between β-lactoglobulin genotypes and milk yield and composition in some Polish sheep breeds. Yousefi et al. (2013) revealed significant associations between AB genotypes and higher milk fat percentage in indigenous Zel sheep. Finally, in Portuguese sheep breeds, a study was conducted to investigate the effect of the genetic variants at the β-lactoglobulin and αS1-casein loci and milk yield. The genetic variants of β-Lactoglobulin was identified by using PCR-RFLP, which in Portuguese sheep breeds β-Lactoglobulin genotype AA was associated with lower milk yield in Serra da Estrela and Merino ewes (Ramos et al., 2009). This marker also affected milk fat content in Serra da Estrela and protein content in Merino. A suggestive effect of the αS1-casein locus on milk yield was detected in Serra da Estrela, but no associations were found between the variants of this marker with milk fat and protein content (Ramos et al., 2009).

Major histocompatibility complex (MHC), an organized cluster of tightly-linked genes, encodes the molecules that bind processed peptide antigens including parasite-derived peptides and presents them to T-lymphocytes, thereby triggering antigen-specific immune responses (Millot, 1978). In sheep, the MHC gene family includes two major subfamilies: class I and class II genes (Klein, 1986). Among sheep MHC class II genes, the expressed DRB1 and DQB1 loci have been found to be highly polymorphic (Woodal et al., 1997; Konnai et al., 2003b; Sun et al., 2004). In particular, a high polymorphism level is present in exon 2, which encodes the antigen-binding site (Outteridge et al., 1996; Konnai et al., 2003a, b). Variation in these genes may impact immune responses to pathogens, which may lead to variation in disease resistance.

The molecular profiles for ewes in high group milk yield show that there are differences between high group ewes with low group ewes in both DNA profile and phenotypic profile, this point can be used by breeder to make or select the parents in future upon these DNA profiles and mate them altogether to increase the allele frequency for marker assisted selection which have high effect on economical traits to increase animals performance in the next generation as well as the outcome of breeder and sheep breeding in Iraqi Kurdistan region.

Conclusion

The molecular and phenotypic results show that there are differences between ewes groups for milk traits ewes in high group produced 355.8 g/ewe/day milk compared with 102.8 g/ewe/day in low group, these values show the ability to increase the breeder outcome about 300% when breeder select the superior animals according to the animal ranking base on molecular and phenotypic profiles. These results showed that there are agreements between BLUP values with DNA sequencing and the selection process with DNA sequencing technique can speed up the improvement and increase milk production in this breed of sheep in Iraqi Kurdistan. For future the bulk sergeant analysis can be performed on various flocks of sheep in Kurdistan which gives more about molecular markers and verification of the result of the present study through selection (and mating) of rams and ewes using the molecular marker investigated in the present study which related to quantitative traits and follow the performance of their progeny in well defined experiment.
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