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Impact of BLG gene polymorphism on some ovine production traits reared in middle part of Iraq

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ABSTRACT

Milk composition and milk yield, considered as a vital role in production, effected by many genes like BLG gene. The current study was aimed to estimate the impact of BLG gene genotyping on production of Iraqi Awassi sheep from 62 ewes via Polymerase Chain Reaction (PCR) then Restriction Fragment Length Polymorphisms (RFLP) method. The high variance in fat (14.24%) but relatively less in lactose (7.66%), protein (7.20%) and SNF (7.91%) that is referred to the possibility of selection of flocks depending on fat percentage. BLG gene was successfully amplified using specific primer. The calculation of allele frequency was done for all samples under study. RFLP with *RsaI* restriction enzyme was revealed three patterns including AA, AB and BB genotypes. AA genotype was appeared at 350bp, AB within 350bp and 450 bp respectively, and BB at 450bp. The dominated genotype was AB (37%) followed by BB (34%) and then AA (29%) with dominant A allele (0.52) than B allele. The deviations probability using Hardy-Weinberg equilibrium (HWE) of BLG gene was based on chi-square (χ^2) test and confirms that all frequencies within Iraqi Awassi population were in HWE ($p < 0.05$). Current study showed that the higher rate of fat yield was related to AA genotype (9.39%), while the lowest to BB (7.84%). The present findings suggested that the significant differences are related between AB genotype (6.71%) and lactose percentage in comparison to another genotypes; AA (5.84%) and BB (6.37%). The density (g cm^{-2}) of milk can be used to determine the quality of milk for all animal breeds. In the present study, the high milk density was related to AB genotyping (1.049%), whereas the low milk density to AA genotype (1.021%). The BLG gene can be incorporated in Iraqi Awassi breeding programs to enhance the development and improve milk components.

Keywords: Iraqi Awassi sheep, BLG gene, Milk component, HWE, Genotyping, RFLP method.
Article type: Research Article.

INTRODUCTION

β- Lactoglobulin is a protein belonging to lipocalin family, which has the ability to bind small hydrophobic molecules (Selvaggi *et al.* 2015). It is absent within milk of human (Sun *et al.* 2018). It is a whey protein in milk of ovine and accounts 17 - 22% approximately from total protein in milk (Selvaggi *et al.* 20015). In addition, β-Lactoglobulin is encoded by BLG gene, which is located on chromosome three in sheep (Hayes & Petit 1993) and exon two, as well as three allelic variances (A, B and C) according to the change of amino acid. Alleles A and B were differing in the position 20 of amino acid (His/Tyr; Moioli *et al.* 2007), whereas, C allele was differing from A allele by exchanging between amino acid at locus 148 (Arg/Gln; Gene Bank Accession No. X12817). Most common of genetic studies on the variants of all sheep breeds reported that the A and B alleles were most common than the other allele (C) which is regarded as rare allele and only found in Black and White Merino sheep breeds/ Carranzana with less frequencies (Selvaggi *et al.* 2015). β- Lactoglobulin are including 162 amino acids and the dimer forms are more stable in milk with molecular weight of 18 kDa than each monomer (Kontopidis *et al.* 2004). Sheep are domesticated animals found in different Iraqi environments and regions. Sheep and goat are called a small ruminant. The sheep (Ovine) have many breeds, however, Awassi is famous sheep breed in Iraq

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and most of the Middle East countries (Jawasreh 2010). It is characterized by a good production of milk and meat. In fact, the milk of sheep and goat was used for production of cheese (Selvaggi & Tufarelli 2012). There are many studies about the relation between genes and the production of ruminants as milk and meat aimed to improve as well as to increase the production of domestic animals (Fadhil 2019a). Therefore, genetic research should be increased on the genes which are responsible for quantitative traits, such as BLG gene and its effects on milk production (Rustempasic, 2018). Moreover, there are many studies suggesting that the polymorphism of BLG gene is helpful as informative marker for milk and its component in sheep (Selvaggi *et al.* 2014; Selvaggi *et al.* 2015; Gras *et al.* 2016; Triantaphyllopoulos *et al.* 2017; Padilla *et al.* 2018, Jawasreh *et al.* 2019; Dakheel *et al.* 2021). There is a relationship between polymorphism of genes and milk yield (and milk composition), therefore the genotyping should be analysed for each breed, before examining and comparing the production and composition of milk (Selvaggi *et al.* 2012). Nowadays, many studies explained the BLG gene polymorphic effect on milk portion, milk fat and milk yield (Padilla *et al.* 2018). Recently, in a study by Dakheel and his colleagues (2021) on BLG gene, three genotype, i.e., AA, BB and AB were observed when examining on milk of Awassi breed in Iraq. Due to the importance of livestock as a food of individuals in the world and using it in import and export processing, many studies are interested on developing and elevating the livestock production (Biradar *et al.* 2012a). The main important dairy products for import and export are fat milk (butter), milk skimmed dry, Ghee and milk whole dried (Verma *et al.* 2012). Moreover, many factors are affecting the production of domestic animals including genetic and environment factors especially heat stress (Biradar *et al.* 2012b). There are also many studies on genetics of organisms (Tonekabony *et al.* 2021; Ramazanova *et al.* 2021; Sharafkhah *et al.* 2022; AL-Lami & Al-Mayaly 2022). Therefore, current study screens some of genetic loci (BLG gene) in Awassi breed to detect the variants within breed and to establish allelic frequency in commercial flocks in order to find the impact of genotyping on milk composition traits in Iraqi Awassi sheep and to improve this type of sheep in Iraq.

MARTIALS AND METHODS

The present study was carried out on 62 individuals (2- 5.5 year olds) of Awassi ewe sheep from many zones of Babylon City in the period of October 1st, 2017 to June 1st, 2018, under natural conditions. The animals were reared with grazing and consuming concentrated ration. The period of milk yield was about 115 days. The daily milk was collected by hand milking from ewe's owner at two times per a day, then kept within specialized tube in ice container to transport to Lab of Food Science College and Public Health lab of Veterinary College, Al-Qasim Green University, Iraq. To determine the amount of milk components per each ewe in every morning and night, lacto flash solution was used to analyse the milk. Five mL blood was obtained from jugular vein of all ewes and kept in EDTA tube in sterilized container under 4 °C and then transported with little shocking and disturbance to Genetic laboratory, Animal Production Department, Agricultural College, Al-Qasim Green University. Thereafter, DNA extraction was performed using Al-Shuhaib (2017) method, then genomic DNA was loaded on 1.5% agarose to detect the DNA bands and stored at -20 °C until the initiation of genetic techniques. From conditions of PCR, 3µL from genomic DNA and primer were mixed with the following components: 10 mM Tris–HCl (pH 9.0), 30 mM KCl, 250 µM of each dNTP "dATP, dGTP, dTTP and dCTP", 1 U Top DNA polymerase and 1.5 mM $MgCl_2$ (BioNeer Company, Korea). One primer was used to amplify the fragment of DNA (Table 1), preparing by Sentibiolab Company, Turkey.

Table 1. Primer sequence, length and fragment size of β-lactoglobulin gene.

Gene	Primer sequence	Orientation	Primer length (bp)
BLG	5`-CAACTCAAGGTCCCTCTCCA-3`	Forward	20
	5`-CTTCAGCTCCTCCACGTACA-3`	Revers	20

*Feligini *et al.* (1998).

Profiling of thermal for LGB gene amplification consisted of initial denaturation, denaturation, annealing, extension and finally the final extension (Table 2).

Table 2. PCR program and the amplification of BLG gene.

Steps	Process	Temp. (°C)	Duration
1	Initial Denaturation	95	10 min
2	Denaturation	94	30 sec
3	Annealing	62	30 sec
4	Extension	72	60 sec
5	Final extension	72	10 min

6	Refrigeration	4	Forever

Restriction Fragment Length Polymorphism (RFLP) technique was used to identify the genotyping by specific restriction enzyme that reveal a pattern difference between the DNA fragment sizes for each organism (Bhattacharya *et al.* 2008). Consequently, the current study was used *RsaI* restriction enzyme (2U μL^{-1}) for hours at 38 °C, to detect the fragment size of DNA between Awassi ewes. Furthermore, beside the restriction enzyme to digest DNA product, gel electrophoreses with about 2% from gel agarose was used, followed by estimating the frequency of alleles and genotype of β- LG gene in Awassi ewe.

Statistical analysis

SAS program (SAS, 2012) was used to detect the results. The data was analysed using Completely Randomized Design (CRD) as many authors such as Khudair *et al.* (2021).

$$Yij = \mu + \beta LGi + Eij$$

where: Yij = the studied characters;
μ: overall mean of Fat%, Lactose% and density (g cm^{-2});
βLGi: fixed effect of the i^{th} pattern at BLG locus (I = AA, Ab and BB).
Eij: random errors with assumption of (no. σ^2).
The genotype and allele frequencies were calculated according to Falconer & Mackay (1996) model.

$$\text{Gene frequency} = \frac{2D + H}{2N}$$

where H = number of heterozygosis animals, D = number of homozygous animals for specific alleles, N= total number of animals.
Chi-square (χ^2) test was used to investigate the deviations from H.W.E and predict the significant difference among animals in population:

$$X^2 = \sum \frac{(\text{Observed No.} - \text{Expected No.})^2}{\text{Expected No.}}$$

General Linear Model (GLM) and the significant impacts among different patterns were tested by Duncan's multiple range Test (Duncan 1955). Mean (μ), standard deviation (σ) and variance (CV%) were calculated using the following model:

$$\sigma = \sqrt{\frac{\sum (x - \mu)^2}{n - 1}}$$

$$\mu = \frac{\sum x}{n}$$

$$CV = \frac{\sigma}{\mu} * 100$$

RESULTS

Descriptive statistics

The calculation of mean, SE and coefficients of variation for milk components are presented in Table 3. Fat percentage are play a vital role in the selection of program due to its use in many manufactures. Therefore, there are many studies highlighted this trait. The analysis of milk components of 86 ewes exhibited the high variance of fat (14.24%), however, relatively less in lactose (7.66%), protein (7.20%) and solid non-fat (SNF; 7.91%) that is referred to the possibility of selection of flocks depending on fat rate (%). These findings were almost similar to those of Fadhil and his colleagues (2019b) who worked on the components of Awassi milk in Iraq. They reported that its lactose, protein and density (g cm^{-2}) were 6.4981%, 4.3681% and 1.0335% respectively, while our results on the values of fat (8.36%) and SNF (11.432%) were inconsistent with those found in their study. These variations possibly arise from the different number of sheep examined in these studies, along with the difference in environmental and many other factors.

3.2 RFLP-PCR assay for BLG gene

DNA isolation using Al-Shuhaib (2017) method was performed. DNA bands were appeared clearly on agarose gel 1.5% (Fig. 1). BLG gene was successfully amplified using specific primer prepared by Sentebiolab, Turkey (Fig. 2).

Table 3. Milk component description in Iraqi Awassi sheep.

Milk components	No. of samples	Mean	SE	CV (%)
Fat (%)	86	5.990	0.191	14.24
SNF (%)	86	10.47	0.096	7.91
Lactose (%)	86	5.680	0.104	7.66
Protein (%)	86	3.831	0.087	7.20
Density g cm^{-2} (%)	86	1.032	0.090	9.23

SE = Standard error; SD= Standard deviation; CV= Coefficient of Variance.

Fig. 1. Genomic DNA of Iraqi Awassi sheep loaded on 1.5% agarose.

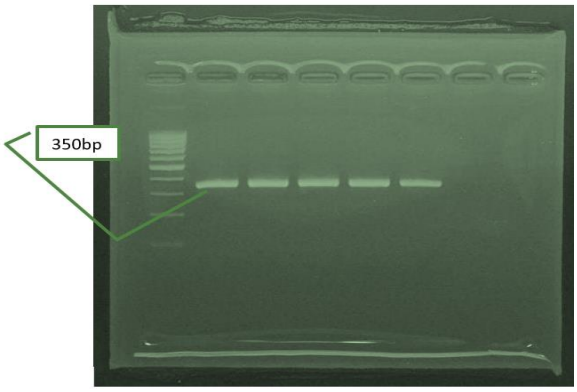

Fig. 2. Amplification of genomic DNA by PCR technique.

Restriction fragment length polymorphism (RFLP) with *RsaI* restriction enzyme revealed three patterns; AA, AB and BB genotypes on 2% agarose gel and DNA ladder (100 bp). AA genotype was appeared at 350 bp, AB genotype within 350bp and 450 bp respectively, and finally BB genotype at 450 bp (Fig. 3).

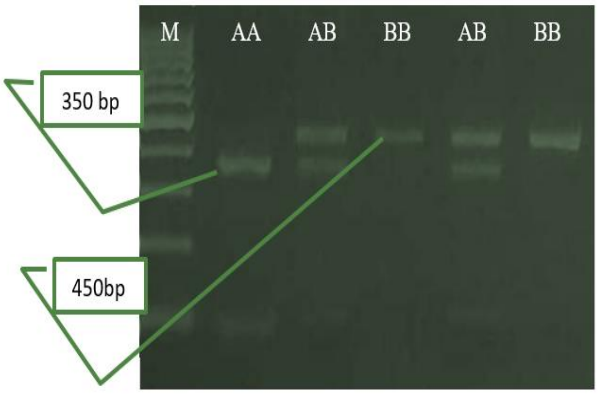

Fig. 3. PCR-RFLP technique of BLG gene on 2% Agarose gel and *RsaI* restriction enzyme. DNA ladder (100 bp).

The dominated genotype was AB (37%) followed by BB (34%) and then AA (29%) with dominant A allele (0.52) than B allele (0.48; Table 4 and Fig. 3), in agreement with the results of Jawasreh *et al.* (2019). They reported in their study on Awassi sheep in Jordan that the allele frequency of B allele (0.58) was higher than A allele (0.42). In addition, the deviations probability using Hardy-Weinberg equilibrium (HWE) of BLG gene was based on chi-square (χ^2) test confirming that all frequencies within Iraqi Awassi population were in HWE ($p < 0.05$; Table 4).

Table 4. Genotypic and allelic frequencies with HWE* of BLG gene in Iraqi Awassi sheep breed.

Awassi sheep (no: 62)	Genotypic			Allele ratio		df**	p. value	χ^2
	AA	AB	BB	A	B			
Number	18	23	21	80	22	2	5.991	$4.14^{n.s}$
Allelic Frequency	0.29	0.37	0.34	0.48	0.52			

Note: HWE = Hardy-Weinberg equilibrium; df = Degree of freedom; ns = Non significant differences ($p > 0.05$); n = number of ewes.

The present study suggested that the higher percentage of fat yield is related to AA genotype (9.39; Table 5). This result is in agreement with Corral *et al.* (2010) who worked on Merino sheep, reporting that the high parentage of fat yield is correlated with AA pattern. On the contrary, the present study was in disagreement with Giaccone *et al*. (2000) who reported that the high parentage of fat yield was related to BB genotype once working on Polish Merino sheep. The lowest percentage of fat yield observed on BB genotyping was 7.84% (Table 4). These differences in fat parentage within breed under study possibly occur due to the variation within environment and feeding of flock as well as a genetic impacts of different genotypes. According to the current study, positive relation was found between genotype AB (6.71%) and lactose in milk composition, in comparison with another genotypes, i.e., BB (6.37%) and AA (5.84%). These findings are in line with the observations reported by Yousefi *et al.* (2013) once working on Iranian Zel sheep, reporting that the highest rate (%) of lactose was in association with AB genotype, in comparison with another genotypes (BB and AA). Moreover, there are many other studies reporting no significant effect between BLG gene and milk compositions (Kaweka & Radko 2011). The density (g cm^{-2}) of milk can be used to determine the quality of milk for all animal breeds. The present study exhibited the correlation of the high milk density with AB genotyping (1.049%), while low with AA genotype (1.021%; Table 5).

Table 5. Milk composition of Iraqi Awassi ewes.

Gene	Genotypes	No.	Least Square means		
			Fat (%)	Lactose (%)	Density (g cm^{-2})
β-LG	AA	18	9.39 ± 1.23^{a}	5.84 ± 0.17^{b}	1.021 ± 0.13^{b}
	AB	23	8.22 ± 0.97^{a}	6.71 ± 0.23^{a}	1.049 ± 0.08^{a}
	BB	21	7.84 ± 0.69^{b}	6.37 ± 0.45^{b}	1.039 ± 0.06^{ab}

Note: a, b and ab in each column are significantly variation ($p < 0.05$).

DISCUSSION AND CONCLUSION

This study reported the relation between BLG gene and milk composition of Iraqi Awassi sheep. BLG gene was selected due to its direct involvement in development and growth of mammary gland to maintain milk synthesis and secretion (Gras *et al.* 2017). The gene under study is located on QTL region that influences milk quality and quantity (Suarez *et al.* 2017). There are different genotypes and allelic frequencies revealed by the present study (Table 4). BLG locus confirms a high genotype frequency of AB (37%) followed by BB (34%) and then AA (29%) with dominant A allele (0.52) than B allele (0.48) in Iraqi Awassi sheep (Table 4). Similar findings were found in Jordanian Awassi breed (Jawasreh *et al.* 2019), Hungarian Awassi sheep (Baranyi *et al.* 2010), Chios sheep (Triantaphyllopoulos *et al.* 2017), Rusty Tsigai sheep (Kusza *et al.* 2015), Racka Sheep (Georgescu *et al.* 2016), Zel sheep (Yousefi *et al.* 2013), and Polish Merino sheep (Kawecka & Radko 2011). In our study, C allele was absent that is probably as a rare allele and detected in few sheep breeds like Turcana, Tsigai, Racka, Karakul of Botosani, Merinoland, Transylvanian Merino, and Hungarian Merino (Selvaggi *et al.* 2015; Kusza *et al.* 2018). In our study, AA genotype of BLG gene was associated with highest fat (%) compared to BB genotype. Significant effects of BLG gene on milk fat (%) of different ovine breeds were reported by previous studies. BLG AA genotype had a significant impact on fat percentage in East Friesian sheep (Giambra *et al.* 2014), Italian Leccese and Altamurana sheep (Dario *et al.* 2008) and Merino sheep (Corral 2010). There is a study on the Spanish Merino sheep conducted by Padilla *et al.* (2018) suggesting that A allele in BLG gene had a positive significant impacts on milk fat (%). There are positive related between genotype AB and lactose and even density (%) in milk composition in comparison with another genotypes, i.e., BB and AA, which is in line with the observations reported by Yousefi *et al.* (2013).

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