## Original study

# Effect of $\beta$ -lactoglobulin and $\kappa$ -casein genes polymorphism on milk composition in indigenous Zel sheep

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# Abstract

The effect of  $\beta$ -lactoglobulin ( $\beta$ -LG) and  $\kappa$ -casein (CSN3) genotypes on milk composition were evaluated in Iranian indigenous Zel sheep breed. Genotypes were determined by PCR amplification followed by digestion with *Rsal* enzyme for exon II of the  $\beta$ -LG gene and SSCP method for exon IV of the *CSN3* gene. Polymorphism was detected in all PCR products.  $\beta$ -lactoglobulin showed two alleles and three genotypes and *CSN3* gene revealed two conformational patterns, respectively. Results indicated that there were significant associations between AB genotype of  $\beta$ -LG gene with higher fat and lactose percentages and also between K<sub>1</sub> pattern of *CSN3* gene with higher lactose percentage. Therefore, it is feasible to improve milk composition in Zel sheep breed using  $\beta$ -LG and *CSN3* genes.

**Keywords:**  $\beta$ -LG, CSN3, milk composition, Zel sheep

**Abbreviations:** β-LG: β-lactoglobulin, CSN3: κ-casein

# Introduction

Because of unique richness of sheep milk and its role in human health, dairy sheep are often practiced alongside the cow milk industry (Haenlein 2006). In dairy sheep industry several limitations make the selection program for increasing milk yield less effective compared to dairy cattle. Lack of widespread use of artificial insemination and standardised milk recording system across flocks are two obvious limitations. Given these limitations, using DNA markers and other genomic tools to increase milk yield is critical for the future of dairy sheep industry) Staiger *et al.* 2010).

Archiv Tierzucht 56 (2013) 21, 216-224 doi: 10.7482/0003-9438-56-021 Received: 22 March 2012 Accepted: 13 June 2012 Online: 8 March 2013

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© 2013 by the authors; licensee Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany. This is an Open Access article distributed under the terms and conditions of the Creative Commons Attribution 3.0 License (http://creativecommons.org/licenses/by/3.0/). The  $\beta$ -Lactoglobulin is the major whey protein in ruminant's milk. The  $\beta$ -lactoglobulin gene is located on ovine chromosome 3 and is one of the specific genes that affect milk traits in sheep (Feligini *et al.* 1998). Several investigations have shown that  $\beta$ -*LG* gene is polymorphic in sheep (Dario *et al.* 2005, Elmaci *et al.* 2006). Three allelic variants of  $\beta$ -*LG* gene consisting A, B and C have been reported in sheep. The A and B variants are present in all breeds, but the C variant is a rare allele found in the Merino breed (Barillet *et al.* 2005). Some works found significant associations between the main variants A and B with milk production (Nudda *et al.* 2000), fat and protein content (Dario et al. 2008).

Casein genes are organised as a tightly linked cluster on ovine chromosome 4 in a 250 kb DNA segment (Bevilacqua *et al.* 2006). The performance of technological processes of cheese production depends on the structure of *CSN3* protein (Zaton 1999).  $\kappa$ -casein plays an important role in the formation, stabilisation and aggregation of the casein micelles thus altering the manufacturing properties and digestibility of milk (Jann *et al.* 2004). The  $\kappa$ -casein polymorphism is well described in cattle (Prinzenberg *et al.* 2008), goat (Jann *et al.* 2004, Prinzenberg *et al.* 2005) and sheep (Ceriotti *et al.* 2004, Coral *et al.* 2010). Among the four casein genes ( $\alpha$ S1-,  $\alpha$ S2-,  $\beta$ -, and  $\kappa$ -casein), *CSN3* is the least studied with respect to its effect on milk yield and composition. One positive association between polymorphism of *CSN3* gene and milk composition has been reported in goat (Chiatti *et al.* 2007).

Determination of the genetic variability of the  $\beta$ -LG and CSN3 genes and their associations with milk composition in Iranian indigenous Zel sheep were the aims of the present study. This intends to be the first step for a deeper study on Zel Sheep breed to establish a breeding program based on these genes.

# **Material and methods**

#### Zel sheep

The Zel breed is a small meat-type sheep kept for both meat and milk production and is the only thin tailed sheep breed in Iran that has a tail with 10-12 cm length, instead of a fat-tail. The main distribution of this breed is on the northern slopes of the Elburz Mountain in the northern provinces of Iran. Lactation in sheep usually lasts about 5 to 5.5 months, but for this breed seven months has been reported. This small sheep has an average body weight of 40-45 kg in mature age and produces an average of 50 kg milk adjusted to 120 lactation days. The daily milk production is approximately 300 ml and is relatively high in fat (8-9%). The coat of Zel sheep can vary from white to black or brown and hands and feet are drawn without wool. The males are horned and the females are polled or naturally hornless. This breed is resistant to hot and humid conditions and parasites (Saadat Noori & Siah Mansoor 1990). It is believed that this breed may be the ancestor of Merino sheep (Mason 1996).

#### Blood samples and DNA extraction

The blood samples were collected randomly from 180 Zel ewes reared in Shirang Research Station, located in Golestan province, Iran. In this station about 230 ewes and 30 rams are reared. DNA was extracted from 100  $\mu$ l of blood, using a commercial kit (Diatom DNA Prep100, ISO Gene, Moscow, Russia) following the manufacturer's protocol. The quality and

quantity of extracted DNA were measured spectrophotometrically and by electrophoresis on 1% agarose gel.

#### Milk samples and analysis

Individual milk samples were taken from 180 lactating Zel ewes. Milk samples were collected in the period of September to December every two weeks from morning milking. Determination of milk composition (fat, protein, lactose, density and non-fat solids) was done using EKOMILK Ultrasonic Milk Analysers (EON trading Inc., Stara Zagora, Bulgaria). All animals were healthy and reared under the same environmental and nutritional conditions. In addition, a synchronization of oestrus was carried out so that the parturitions were occurred at the same time.

#### DNA amplification by PCR

Polymerase Chain Reaction (PCR) was carried out, using the Personal Cycler thermocycler (Biometra, Goettingen, Germany) and the PCR Master Kit (Cinna Gen Inc., Tehran, Iran). The kit of master mix consisted of 0.04 U/µl of *Taq* DNA polymerase, 10×PCR buffer, 3 mM MgCl<sub>2</sub> and 0.04 mM dNTPs (each). Each reaction mixture consisted of 12.5 µl of the master mix, 1 µl of the DNA solution (50 to 100 ng/µl), 1 µl of each primer (5 pmol/µl) and some deionized water making up a final volume of 25 µl.

For amplifying a 452 bp region from exon II of  $\beta$ -LG gene, primers described by Eignatev (1998) were used:

BLG- F (5'-TTG GGT TCA GTG TGA GTC TGG-3')

BLG- R (5'-AAA AGC CCT GGG TGG GCA GC-3')

The amplification reaction was carried out under the following conditions:

An initial denaturation step at 95 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 45 s and extension at 72 °C for 45 s, and a final extension of 72 °C for 10 min.

Amplification for a 416 bp fragment from the exon IV of the ovine *CSN3* gene was carried out using primers suggested by Barracosa (1996) under the following conditions:

κ-CSN- F (5'-GAG AAA GAT GAA AGA TTC TTC G-3')

κ-CSN- R (5'-GCT TCT GGA TTA TCT ACA GTG-3')

An initial denaturation at 95 °C for 5 min followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 8 min.

The PCR correctness was assessed by electrophoresis of each sample (4  $\mu$ l) on 1.3 % (w/v) agarose gel.

#### PCR-RFLP

β-Lactoglobulin gene variants were identified by PCR-RFLP method. Samples (8 μl) of each PCR product were incubated for 16 h at 37 °C with 5 U *Rsal* enzyme, according to manufacturer's instructions (Fermentas, Vilnius, Lithuania). Digestion products were separated by electrophoresis on 3 % agarose gel in 1×TBE buffer. The gels were run at 80 V for one hour and visualized under ultraviolet light after staining with ethidium bromide.

#### PCR-SSCP

 $\kappa$ -casein gene variants were identified by PCR-SSCP method. For SSCP analysis, 5 μL of each amplification product was added to 15 μL of denaturising solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were heat-denatured at 95 °C for 5 min, immediately chilled on ice and loaded onto 12% polyacrylamide gel (39:1). The gels were run at 280-300 V for 12-14 h at 4°C. The electrophoresis was carried out in a vertical unit in 1×TBE buffer. The gels were stained with silver nitrate to observe the conformational patterns.

## Statistical analysis

Allele and genotype frequencies were calculated using Pop-Gene, 1.31 software (Yeh *et al.* 1997). A chi-square ( $\chi^2$ ) test was performed to test the goodness of fit to Hardy-Weinberg equilibrium expectations for the distribution of genotypes. In order to test the association of different genotypes of the studied genes with milk composition, statistical analysis was performed using mixed procedure of the SAS program and least squares means of the genotypes were compared by the Tukey test (SAS 1999). The following model was used:

$$Y_{iikl} = \mu + G_i + R_i + (GR)_{il}P_k + TW_l + b(BW - \overline{BW}) + e_{iikl}$$
(1)

where  $Y_{ijkl}$  is the dependent variable considered (milk parameters consisting percentage of fat, protein, lactose, solids-not-fat and density),  $\mu$  is the overall mean,  $G_i$  is the fixed effect of the ith genotype or conformational pattern (*i*=1,...,3),  $R_j$  is the fixed effect of the *j*-th test day record (*j*=1,...,7), (*GR*)<sub>ij</sub> is the interaction effect of genotype and test day record,  $P_k$  is the fixed effect of the *k*-th parity number (*k*=1,...,3), class 3 included ewes in third and over parities,  $TW_i$  is the fixed effect of the *l*-th lambing type (*l*=1, single and 2, twin),  $b_i$  is the linear regression coefficient of body weight, *BW* is the body weight of each ewe, *BW* is the mean body weight of ewes and  $e_{ijkl}$  is the random residual error. BW of each ewe was considered as covariate in the model.

## Results

#### Gene and genetic frequencies

Digestion of the 452 bp fragment of  $\beta$ -LG with Rsal restriction enzyme, revealed alleles A and B, and three genotypes with different sizes consisting AA (175, 170, 66 and 41 bp, AB (236, 175, 170, 66 and 41 bp and BB (236, 175 and 41 bp) (Figure 1). The PCR-SSCP for CSN3 gene was carried out on polyacrylamide gel and two (K<sub>1</sub> and K<sub>2</sub>) different conformational patterns were observed (Figure 2).

The frequencies of  $\beta$ -LG alleles and genotypes and banding patterns of CSN3 gene are presented in Table 1.

Table 2 shows the expected, observed and average heterozygosities, fixation index ( $F_{IS}$ ) and the Hardy-Weinberg equilibrium value of  $\beta$ -LG gene in the studied sheep. Results showed a significant difference between the observed and expected frequencies of  $\beta$ -LG gene and didn't confirm the Hardy-Weinberg equilibrium. Observed heterozygosity for this locus was very low, showing a low degree of heterozygosity and genetic variability in this locus.



Figure 1

RFLP analysis of the 452 bp fragment of  $\beta$ -LG gene on 1.5 % agarose gel.



Figure 2 SSCP analysis of the 416 bp fragment of  $\kappa$ -Cn gene on 12% polyacrylamide gel.

Table 1

Distribution of  $\beta$ -LG alleles and genotypes and banding patterns frequency of CSN3

Method	Gene	Number	Genotype or Pattern	frequency	Allele	Frequency
RFLP	β-LG	16	AA	0.089	А	0.14
		16	AB	0.089	В	0.86
		148	BB	0.822		
SSCP	CSN3	95	K1	0.528		
		85	K2	0.472		

Table 2

Expected (He), observed (Ho) and Average (Ave-He) Heterozygosities, Fixation index ( $F_{IS}$ ) and HWE value obtained for  $\beta$ -LG gene

Gene	Не	Но	Ave-He	F <sub>is</sub>	HWE
β-LG	0.232	0.089	0.231	0.614	69.7

#### Genetic association with milk composition

In this study, the association between the genetic polymorphisms of  $\beta$ -LG and CSN3 gene with qualitative characteristics of milk were investigated. According to statistical analysis, significant associations (*P*<0.05) were found between AB genotype of  $\beta$ -LG gene with higher percentages of fat and lactose. K<sub>1</sub> pattern of CSN3 gene also showed significant associations with higher percentages of lactose and milk density (*P*<0.05). However, other genotypes of  $\beta$ -LG gene and conformational patterns of CSN3 gene showed no associations with other milk components (Table 3). Means and standard deviations of the dependent variables are shown in Table 3.

Table 4 shows the tested of the statistical significance of fixed effects considered in the mixed model used to analyse the traits under study. According to these results, lambing type and test day record had significant effects on protein, lactose, density and solids-not-fat. In addition, the ewes having two lambs and the last test day record had more effect on these traits.

Parame	eter					
Gene	Genotype or pattern	Fat, %	Protein, %	Lactose, %	SNF, %	Density, g/cm <sup>3</sup>
		Mean=6.65 SD=2.18	Mean=5.73 SD=1.15	Mean=4.47 SD=0.116	Mean=11.06 SD=1.39	Mean=1.035 SD=0.02
β-LG	AA	6.28±0.70 <sup>b</sup>	6.90±0.30	4.60±0.05 <sup>b</sup>	12.50±0.38	1.0383±0.006
	AB	$8.00 \pm 0.70^{\circ}$	6.78±0.30	4.70±0.05°	12.33±0.38	1.0374±0.006
	BB	6.87±0.40 <sup>b</sup>	6.85±0.20	4.56±0.03 <sup>b</sup>	12.42±0.22	1.0391±0.004
CSN3	К,	6.70±0.42	6.90±0.18	4.60±0.02ª	12.47±0.23	1.0384±0.004
	K,	7.00±0.45	6.75±0.18	4.53±0.02 <sup>b</sup>	12.23±0.23	1.0387±0.004

Table 3 LSmeans $\pm$ standard errors of the milk composition for the  $\beta$ -LG genotypes and CSN3 patterns

LSmeans with different letters (a and b) in each colum are significantly different (P<0.05).

#### Table 4

P-values obtained for each fixed factor considered in the mixed model used to analyse the traits under study

Factor	Fat, %	Protein, %	Lactose, %	SNF, %	Density, g/cm <sup>3</sup>
lambing type	0.73	<0.0001	0.0004	< 0.0001	0.25
test day record	0.79	<0.0001	< 0.0001	< 0.0001	0.03
parity	0.09	0.13	0.81	0.39	0.83
body weight	0.72	0.31	0.69	0.64	0.57

## Discussion

#### β-lactoglobulin gene

The distribution of allele frequencies of  $\beta$ -*LG* gene showed that the frequency of allele B (0.86) was higher than that of allele A (0.14) in Zel breed. Similar results were reported in Awassi breed by Baranyi *et al.* (2010). Also the B allele was detected to be at high frequency by several studies (Wessels *et al.* 2004, Mroczkowski *et al.* 2004, Barillet *et al.* 2005, Staiger *et al.* 2010, Arora *et al.* 2010). Nevertheless, Baranyi *et al.* (2010) reported a higher frequency of allele A than that of allele B in Racka sheep. Also, (Mohammadi *et al.* 2006, Mele *et al.* 2007, Dario *et al.* 2008, Michalcova & Krupova 2009, Corral *et al.* 2010) reported that allele A is dominant in different sheep breeds.

Statistical analysis revealed significant associations between AB genotype of  $\beta$ -LG gene with higher percentages of fat and milk lactose. These results may be explained by the fact that  $\beta$ -LG protein plays an important role in transport and metabolism of fatty acids (Frapin *et al.* 1993). Present results are in agreement with previous reported by Ramos *et al.* (2009) and Dario *et al.* (2008) who reported an association between AB genotype and fat percentage of milk in Serra da Estrela and Leccese dairy ewes, respectively. Mele *et al.* (2007) reported that AB genotype of  $\beta$ -LG gene is associated with milk fatty acid in Massese sheep. Celik & Özdemir (2006) reported that AB genotype is associated with higher lactose content in Awassi Sheep. An association of  $\beta$ -LG AB with higher lactose and fat yield has also been reported in Holstein cow (Tsiaras *et al.* 2005). Genotypes of  $\beta$ -LG gene were not significantly associated with other milk components. This is in agreement with several studies (Sumantri *et al.* 2008, Michalcova & Krupova 2009). Kawecka & Radko (2011) found no statistical associations between  $\beta$ -LG genotypes and milk yield and composition in Polish Mountain, East Friesian, Polish Merino and Austrian Bergschaf sheep. However, most of the studies have reported significant associations between  $\beta$ -LG polymorphism and milk related traits (Cubric-Curik *et al.* 2002, Corral *et al.* 2010).

#### CSN3 gene

Despite the crucial role of CSN3 in the micelles structure and the clotting process, very few studies were done with respect to its effect on milk composition. In this current study, two conformational patterns (K, and K,) with relatively equal frequencies were identified for the CSN3 gene. Our results at the CSN3 locus were similar to those reported by Ceriotti et al. (2004) and Ahani Azari et al. (2011) in which the PCR-SSCP analysis of exon IV of this gene showed two conformational patterns in three Italian sheep breeds and Dalagh sheep, respectively. Barracosa (1996) also found two conformational patterns in Serra da Estrela sheep. However, other researchers observed different polymorphisms (Feligini et al. 2005, Staiger et al. 2010), or even monomorphism (Bastos et al. 2001, Mandal et al. 2008, Giambra et al. 2010) for this locus in different sheep breeds. Analysis of milk traits revealed significant associations between K, pattern of CSN3 gene with higher lactose percentage compared to K, pattern (P < 0.05). These effects are probably due to these facts that this protein is a glycopeptide and there is a carbohydrate structure only in this casein among other caseins (Masoudi 1998). No significant associations were found between conformational patterns of CSN3 gene and fat, protein, density and solids-non-fat. These results are in agreement with Staiger et al. (2010) who studied a Rsal polymorphism and found no significant effect of CSN3 polymorphism on milk production.

The inconsistency of the present results compared to other studies may be ascribed to breed differences, population size, sampling, environmental factors and frequency distribution of genetic variants.

Because of very few studies on *CSN3* polymorphism using SSCP method and their effects on milk production traits in sheep, the comparison of present results with other studies was not completely possible. This paper tried to give new and more information on polymorphisms of  $\beta$ -LG and *CSN3* genes in Zel Sheep breed which eventually provide useful information for breeding program based on marker-assisted selection.

In conclusion, the results of the present study indicated significant associations between AB genotype of  $\beta$ -LG gene and higher fat and milk lactose percentages and between K<sub>1</sub> pattern of CSN3 gene and higher percentage of milk lactose. Although present results agreed with some previous studies, further studies with larger numbers of animals are necessary to confirm the associations between these genes and milk production traits. Furthermore, our results showed that PCR-RFLP and PCR-SSCP are appropriate tools for detecting polymorphism and evaluating genetic variability.

## Acknowledgements

This work was financially supported by the Agriculture of Jahad (Golestan, Iran). The authors also acknowledge the staff of Shirang Research Station for their help in providing milk and blood samples.

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