Research Article

Allelic polymorphism of *K-casein*, *β-Lactoglobulin* and *leptin* genes and their association with milk production traits in Iranian Holstein cattle

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Abstract

The purpose of this study was to investigate the polymorphism of *K-casein* (*K-CN*), *β-Lactoglobulin (B-LG*) and *leptin* (*LP*) genes in Iranian Holstein cattle by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. DNA was extracted from blood samples of 139 cows using a modified phenol chloroform method. Association between *K-CN*, *B-LG* and *LP* genes' polymorphism with milk production traits were investigated using mixed procedure of SAS software. The frequencies of AA, AB and BB genotypes for *K-CN* (0.72, 0.18 and 0.10), *B-LG* (0.43, 0.28 and 0.29) and *LP* (0.24, 0.63 and 0.13) were also calculated. Statistical results revealed a significant association between AA and BB genotypes of the *K-CN* gene with milk production and milk protein percentage, respectively. Also, BB genotype of the *B-LG* gene and AA genotype of the *LP* gene showed a significant association with protein percentage and milk production ($P<0.05$), respectively. Therefore, it is feasible to improve milk production traits in these herds using the studied genes.

***Keywords*:** Holstein cattle, *K-casein, β-Lactoglobulin, leptin* gene, PCR-RFLP, milk production traits

Introduction

Genotypes that affect milk production traits could be used as genetic markers in marker-assisted selection programs. Although genes with large effects only show part of the genetic differences between animals, identifying these genes could be a great help to estimate more accurate breeding values in breeding programs (Hayes et al., 2009). Many studies have been conducted on milk protein polymorphisms to determine frequencies of genetic variants and their associations with milk production traits (Erhardt, 1996; Vohra et al., 2006). Milk proteins in ruminants are divided to two main groups named caseins and whey proteins. *B-LG* is the main part of the whey proteins in ruminant species, and its locus is located on chromosome 11 (Simpson et al., 1998). This gene is very polymorphic and has about 12 alleles (Godovac-Zimmermann et al., 1996). The most frequent ones are A and B variants that are the results of point mutations in exon 4 of the *B-LG* gene leading to two amino acid changes at positions 64 (Asp→Gly) and 118 (Val→Ile) (Rachagani et al., 2006). It has been shown that high protein content in ruminant's milk with AA genotype is due to more *B-LG* protein in their milk (Ng-kwai-hang, 1998; Glantz, 2011). *K-CN* is a polymorphic gene in cattle that has 5 exons and 4 introns. This gene has several forms and 13 alleles have been reported for this gene so far (Prinzenberg et al., 2008). The most common alleles are A and B. A is different from B because of substitution of two amino acids at positions 136 (Thr → Ile) and 148 (Asp → Ala) (Grosclaude et al., 1972). The effect of A and B forms on milk traits in cattle have been widely studied and most of the results suggest a positive effect of BB genotype compared with AA genotype on milk traits such as fat and milk protein (Lunden et al., 1997).

Leptin is a hormone that is involved in the regulation of food intake, long-term energy balance, body weight, reproductive and immune system. Leptin is encoded by the OB gene that has three exons and is located on chromosome 4. Recently, polymorphisms have been reported for this gene suggesting that it as a candidate affecting the milk production and its components (Buchanan et al., 2002; Buchanan et al., 2003). Due to the fact that nearly all of the dairy cattle in the industrial dairy farms in Iran are Holstein, this study aimed at investigating *K-CN*, *B-LG* and *LP* polymorphisms in Iranian Holstein cattle and their associations with milk production and components.

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Materials and Methods

Blood samples were collected from 139 Holstein cows belonging to six different dairy farms participating in the recording system of National Animal Breeding Center. DNA was extracted from whole blood using modified phenol chloroform method. In order to determine the quantity and quality of the extracted DNA, both spectrophotometric and gel monitoring methods were used. Optimization of PCR conditions were mainly done on three important factors including concentration of $MgCl_2$, PCR primer binding temperature and PCR temperature program. PCR reactions containing 50 ng of genomic DNA, 200 mM of each dNTPs, 25 mM of each primer, 2.5 mM $MgCl_2$, and 1 unit of Taq DNA polymerase enzyme, were carried out in a final volume of 15 µl. The 247 bp fragment, comprising a part of the IV exon and intron of the genomic DNA was amplified by using primers as suggested by Strazalkowska et al. (2002). A 422 bp fragment of intron 2 in bovine leptin gene was amplified by PCR using forward and reverse primers according to Liefers et al., (2002) and the primers used for amplification of κ-casein gene fragment were reported by Mitra et al., in 1998. The primers used for amplification are given in Table 1.

For amplification of *K-CN* gene, an initial denaturation step at 95°C for 4 min followed by 35 cycles of denaturation at 94℃ at 30 seconds, annealing at 58℃ at 30 seconds and extension at 72°C for 45 sec, and a final extension of 72°C for 5 min were set. PCR amplification programs for the *B-LG* and *LP* genes were similar to those of the *K-CN*, but the reactions were carried out at 60℃ as annealing temperature and synthesis step for *B-LG* was set at 70℃. PCR reactions were carried out using CG1-96 thermocycler (Corbet). The PCR products were visualized using electrophoresis on 2% agarose gel in 0.5X TBE buffer then 5 µl of *B-LG* and *K-CN* PCR products were digested overnight at 37°C with 3 units of Hinf1 and HaeIII enzymes overnight at 37°C, respectively. The *LP* PCR products were digested with 3 units of *Kpn*2I restriction enzyme at 55℃ overnight. The digested products were separated on 3.5% agarose gel and detected with ethidium bromide staining. Allele scoring and counting were performed by comparing with the standard markers.

In order to perform association analysis, 2532 test day records, including production of milk protein, milk fat and milk somatic cell counts (SCC), collected during 2001-2006 from six Iranian Holstein herds were used. Determination of milk composition (fat, protein and somatic cell counts) was done using Ultrasonic Milk Analyzers (EKOMILK, Bulgarian). After Kolmogorov-Smirnov test on the data and insurance of the normality of the data, traits were included in the statistical model. Population genetic parameters such as allele and genotype frequencies, expected heterozygosity (H_E) and observed homozygosity (H_0) were calculated using PopGen 2.32 software. Likelihood ratio test ($G2_T$) was used to determine the Hardy-Weinberg equilibrium status of the loci (Guo and Thompson, 1992). In order to calculate the effect of different genotypes on milk production traits, a mixed model in accordance with repeated measurements was considered. Statistical analysis was performed based on Mixed Proc using SAS software (SAS software, 2002). The following model was used to analyze the association between milk yield, fat, protein and somatic cells counts with studied genotypes:

$$Y_{ijklmno} = \mu + P_i + S_j + H_k + T_l + G_m + R_{ijklm} + e_{ijklmno}$$

where $Y_{ijklmno}$= milk production, milk fat, milk protein or somatic cell score, µ= overall mean of the trait under analysis, P_i= effect of lactation with 6 classes (cows with 6 and more lactations were merged in one class), S_j= the calving season effect with four classes, H_k= fixed effect of herd with 6 classes, T_l= fixed effects of year with 5 classes, G_m= fixed effect of studied genes, $R_{ijklm=}$ the random permanent cow effect with mean 0 and variance S^2_{δ}, and finally $e_{ijklmno}$ is total residual effects with mean 0 and variance σ^2.

Table 1. Primers used for PCR amplification of of the experiment.

Locus	Sequence
K-CN locus	Forward Primer: 5′- ATC ATT TAT GGC CAT TCC ACC AAA G-3′ Reverse Primer: 5′- GGC CAT TTC GCC TTC TCT GTA ACA GA-3′
B-LG locus	Forward Primer: 5′- TGT GCT GGA CAC CGA CTA CAA AAAG-3′ Reverse Primer: 5′- GCT CCC GGT ATA TGA CCA CCC TCT-3′
LP locus	Forward Primer: 5′-ATG CGC TGT GGA CCC CTG TAT C-3′ Reverse Primer: 5′-TGG TGT CAT CCT GGA CCT TCC-3′

Results

The specific primers for the *K-CN* gene amplified a fragment corresponding to the exon 4 and intron 4 with a size of 350 bp. digesting this fragment by *Hinf1* enzyme produced three different

patterns. The first pattern produced two bands (226 and 84 bp), the second pattern produced three bands (134, 132 and 84) while the third pattern produced four different bands (226, 134, 132 and 84 bp) (Fig. 1). The AB genotype showed all four possible bands. The allelic frequencies reported in the studies for *K-CN* was similar to Lundén *et al*. (1997) and Cardak (2005).

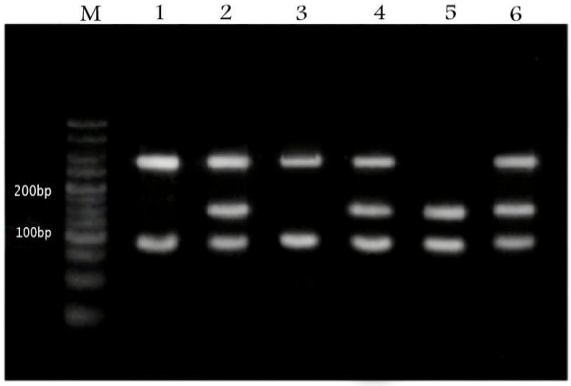

Figure 1. The gel electrophoresis pattern of the fragments produced from PCR on *K-CN* genes and restriction digested with *HinfI* enzyme. Lane 2, 4 and 6, represents AB, lane 1 and 3 represent BB and lane 5 represent AA genotypes and M is a 50 bp ladder marker.

The specific *B-LG* primers amplified a 247 bp fragment corresponding to its exon 4. Digestion of this fragment by *Hae*III showed three patterns. In the first pattern, two bands of 148 and 99 bp (AA genotype), in the second pattern two bands of 99 and 74 bp (BB genotype) and in the third pattern three bands of 148, 99 and 74 bp (AB genotype) were observed (Fig. 2). The allelic frequency for *B-LG* that reported by Tsiaras *et al*. (2005) and Hill (1993), confirm the frequencies obtained in current study.

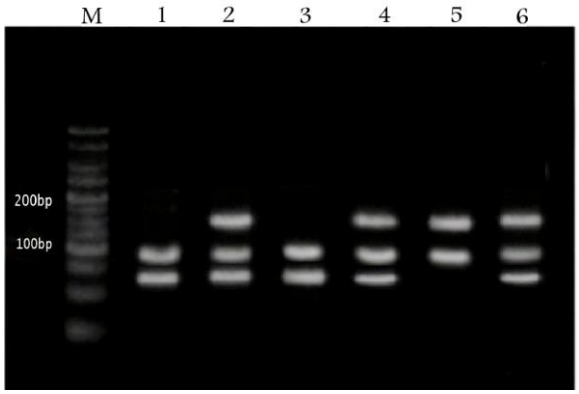

Figure 2. Pattern of *B-LG* gene after treatment with *HaeIII* enzyme. Lane 1 and 3 represent BB, lane 2, 4 and 6 represent AB and lane 5 represent AA and M is a 50 bp ladder marker.

The specific *LP* primers amplified a 94 bp fragment of its exon 2. Digestion of this fragment by *Kpn*2I showed three patterns. In the first pattern, a 94 bp band (AA genotype), in the second pattern two bands with the size of 19 and 74 bp (BB genotype) while in the third pattern three bands with the size of 19, 74 and 94 bp (AB genotype) were observed (Fig. 3). Similar studies by Moravčíková and et al. (2012) on Slovakian cattle and Buchanan *et al*. (2003) on Angus and Herford cattle, revealed that the predominant allele was A. In both populations, they observed all three genotypes AA, AB and BB, as we did in current study.

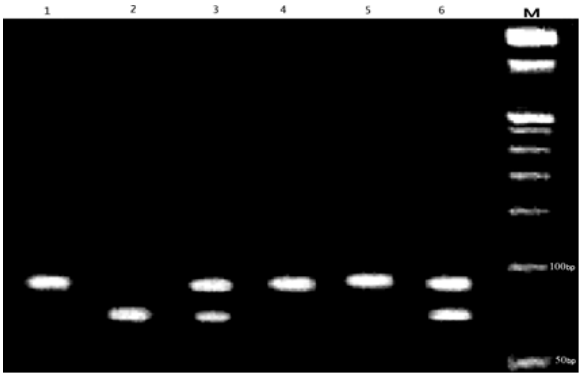

Figure 3. The gel electrophoresis pattern of fragments produced from PCR on *LP* gene after treatment with *Kpn2I* enzyme. Lane 1, 4 and 5 represent AA, lanes 3 and 6 represent AB and lane 2 represent BB and M is a 50 bp ladder marker.

Allele frequency obtained in the present study was in consistence with the previous reports. However, in some cases, may be for the reasons like different statistical designs, i.e type of continuous variables (for example, breeding values, average deviation of daughter performance or production records), different calving stage and environmental conditions, this consistency was not observed.

Table 2. Gene and genotype frequencies for K-casein, β-Lactoglobulin and leptin genes in Iranian Holstein cattle determined by PCR-RFLP.

Loci	Gene frequency		Genotype frequency			H_E^*
	A	B	BB	AB	AA	
K-CN	0.81	0.19	0.1	0.18	0.72	0.31
B-LG	0.57	0.43	0.29	0.28	0.43	0.49
LP	0.61	0.39	0.13	0.63	0.24	

* H_E: Expected Hetrozygosity

The gene and genotype frequencies for *K-CN*, *B-LG* and *LP* loci and the expected heterozygosity are summarized in table 2. Based on the results of likelihood ratio test, all of the loci were not in Hardy-Weinberg equilibrium (P<0.05). This might be due to the deviation of the population from the Hardy-Weinberg equilibrium conditions such as existence of selection, genetic drift, small size of the population, etc. The allele frequencies observed in this study is fully consistent with previous reports (Buchanan *et al.*, 2003; Prinzenberg *et al.*, 1996; Sharifzadeh *et al.*, 2012; Soria *et al.*, 2003).

Table 3. Statistical analysis and probability values of the tested loci

Trait	*K-CN*	B-Lactoglobulin	*LP*
milk production	0.040*	0.215	0.030*
fat percentage	0.414	0.594	0.122
protein percentage	0.075*	0.007*	0.113
somatic cell score	0.613	0.022	0.546

* Statistically significant at the probability level (P < 0.05).

Table 4. Comparison of the least square means and standard errors of milk production traits of different genotypes.

Genotype	milk production	milk protein %	milk fat %	milk somatic cell score
		K-CN		
AA	29.08±7.36[a]	3.09±0.27[b]	3.44±0.53	3.31±1.05
AB	28.11±5.89[b]	3.19±0.13[b]	3.36±0.29	3.331±0.68
BB	28.03±6.17[b]	3.20±0.36[a]	3.39±0.31	3.27±0.73
		B-Lactoglobulin		
AA	25.58±5.79	3.11±0.38[b]	3.40±0.59	3.35±0.92
AB	28.76±8.08	3.16±0.19[b]	3.39±0.43	3.26±1.11
BB	28.74±7.34	3.27±0.27[a]	3.46±0.39	3.27±0.89
		LP		
AA	29.74±52.13[a]	3.15±0.18	3.20±0.39	3.32±0.5
AB	24.12±52.33[b]	3.16±0.10	3.25±0.23	3.36±1.00
BB	23.91±38.66[b]	3.17±0.12	3.23±0.39	3.37±0.90

LS means with different letters (a, and b) are significantly different (P < 0.05).

Undoubtedly, imported sperms from countries such as America, Canada, Australia and some European countries and their use in Iranian cattle industry affected this consistency. Tables 3 and 4 summarize the results of statistical analysis and least square means for different genotypes and traits. Results from analysis of variance, indicated significant herd and lactation effects on fat percentage, milk production and milk somatic cell (P<0.05). Also, *K-CN* locus had a significant relationship with milk production (P<0.05) and protein percentage (P<0.07). Comparison of the least square means showed significant difference between AA and BB genotypes of the *K-CN* gene for milk production and milk protein percentage. However, polymorphisms of this locus were not associated with milk fat percentage and milk somatic cell score. These results were confirmed by Tsiaras et al. (2005) that reported B variant of κ-CN gene has a favorable effect on protein yield. There are also some studies that failed to find any relationships between κ-CN genotypes with production traits (Hamza et al., 2011).

The BB genotype of the *B-LG* gene has shown a strong relationship with protein percentage (P<0.01) while the *B-LG* gene had no association with milk production, fat percentage and somatic cell score. It has also been shown that high protein content in ruminant's milk, with AA genotype, is due to more *B-LG* protein in their milk (Ng-kwai-hang, 1998; Glantz, 2011). In contrast of current results, several authors have reported no significant associations of genotypes on milk yield (Tsiaras et al., 2005). However, reports exist where β-LG genotypes have been positively associated with milk yield (Cardak, 2005; Heidari, 2009). The AA genotype of β-LG has also been shown to have a favorable effect on protein yield (Cardak, 2005; Bovenhuis *et al*., 1992). Also, there are only a few studies in literature trying to find an association between the polymorphism of κ-CN and β-LG with milk somatic cell count records. As one of the rare studies on this subject, Lunden et al., (1997) reported no association between the polymorphism of κ-CN and β-LG with milk SCC. This observation is in coincidence with the result of the current study.

Discussion

The results shows a significant association between AA genotypes of the *LP* gene milk production (P<0.05). Also, the same result was observed by Moravčíková and et al., (2012) in pinzgau cows with AA genotype on milk and protein yield.

Undoubtfully, the distribution of Holstein semen worldwide and using of imported semen extensively in the country farms has resulted in the complete accordance with the outcomes of the forementioned investigations. Possible divergence from Hardy-Weinberg expectations at each locus was evaluated. Both loci showed significant deviation ($p<0.05$) from Hardy-Weinberg proportions. These results were expected, because a number of basic underlying assumptions of Hardy-Weinberg equilibrium such as random mating, infinite population size and equal parental contribution in a breeding population were violated. In conclusion, the findings of this study indicated the existence of polymorphism in all of the three loci responsible for milk yield and compositions. These findings are in consistent with some of the previous studies. The relative consistency can be due to the global distribution of sperm produced in the Holstein breed around the world. Incorporating of the identified direct markers, linked to milk yield and composition to the breeding program for Holstein cattle in Iran can lead to a more rapid genetic progress for milk yield and composition.

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