

Original Research

Allele specific-PCR and melting curve analysis showed relatively high frequency of β -casein gene A1 allele in Iranian Holstein, Simmental and native cows

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Abstract: There are two allelic forms of A1 and A2 of β -casein gene in dairy cattle. Proteolytic digestion of bovine β -casein A1 type produces bioactive peptide of β -casomorphin-7 known as milk devil. β -casomorphin-7 causes many diseases, including type 1 diabetes, cardiovascular disease syndrome, sudden death and madness. The aim of the present study was to determine the different allelic forms of β -casein gene in Iranian Holstein, Simmental and native cattle in order to identify A1 and A2 variants. The blood samples were collected randomly and DNA was extracted using modified salting out method. An 854 bp fragment including part of exon 7 and part of intron 6 of β -casein gene was amplified by allele specific polymerase chain reaction (AS-PCR). Also, the accuracy of AS-PCR genotyping has been confirmed by melting temperature curve analysis using Real-time PCR machinery. The comparison of observed allele and genotype frequency among the studied breeds was performed using the Fisher exact and Chi-squared test, respectively by SAS program. Obtained results showed the A1 allele frequencies of 50, 51.57, 54.5, 49.4 and 46.6% in Holstein, Simmental, Sistani, Taleshi and Mazandarani cattle populations, respectively. The chi-square test was shown that no any populations were in Hardy-Weinberg equilibrium for studied marker locus. Comparison and analysis of the test results for allelic frequency showed no any significant differences between breeds ($P > 0.05$). The frequency of observed genotypes only differs significantly between Holstein and Taleshi breeds but no any statistically significant differences were found for other breeds ($P > 0.05$). A relatively high frequency of β -casein A1 allele was observed in Iranian native cattle. Therefore, determine the genotypes and preference alleles A2 in these native and commercial cattle is recommended.

Key words: β -casein, AS-PCR, Holstein, Simmental, Native cattle, Bioactive peptide.

Introduction

The milk proteins and its products, comparing to other protein from animal or plant resources, have a high nutrient value. There are two groups of large proteins of caseins and whey in milk, in which caseins form a major proportion. Casein genes are a set of four consecutive genes located on chromosome 6q31–33 in cattle and chromosome 4 in sheep and goat. The casein genes divided to four major groups of K -casein (molecular weight 19800 Dalton and 169 amino acids), β -casein (a molecular weight of 2400 Dalton and 209 amino acids), $\alpha S1$ -casein (a molecular weight of 2300 Dalton and 199 amino acids) and $\alpha S2$ -casein (a molecular weight of 2500 Dalton and 207 amino acids) (1-4). In all mammalian species the casein genes are more heterozygous than whey, as β -casein showed 13 alleles (5). It seems that a $S1$ -, a $S2$ - and β -casein genes encoding casein sensitive to calcium, which is segregated from one ancestral gene (6). Thirteen different types of β -casein alleles containing A1, A2, A3, B, C, D, E, F, H1, H2,

I, G and A4 has been reported in Korean native cattle (7). Also there is gamma-casein that is produced from degradation of β -casein (8). Different allelic forms of β -casein gene including A1 and A2 have been reported in most of dairy cattle. These two alleles are different at position 67 of the β -casein chain, in which proline in variant A2 is substituted by histidine in variant A1 (4, 9). The A1 and A2 variants are remained constant during the milk processing. In digestive tract the β -casein A1 be digested differently than the A2 and releases β -casomorphin-7 peptides, which is a small part of the β -casein protein. β -casomorphin-7 (Tyro-Pro-Phe-Pro-

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Gly-Pro-Ile) is detected as a compound with morphine-like activity (10) and also was named as the "milk devil" or casomorphin which means casein and morphine. This substance has characteristics like morphine and if enters the bloodstream through the stomach, causing a variety of problems. It does not want to claim the body and depend the specific genetic makeup of a person causes a variety of diseases such as imbalanced blood-brain barrier (11).

Bioactive peptide β -casomorphin-7 is produced by proteolytic digestion of A1 β -casein of cow milk in vitro (12). The effect of β -casein A1 on type 1 diabetes and its association with other cardiovascular diseases were studied in an experiment. The results showed a strong correlation between the use of β -casein A1 and the incidence of type 1 diabetes ($r^2=0.84$). Also it is reported that high consumption of milk (β -casein A1 type) will increase the diabetes, risk of heart disease and diabetes in children (11). Furthermore, the β -casomorphin-7 caused schizophrenia and itself retaining. β -casomorphin-7 has also been reported widely in the urine of hallucinatory (11). A research was conducted on β -casein gene in Polish Holstein bulls. Results showed that A1 β -casein digestion produced bioactive peptide of β -casomorphin-7 that causes of cardiovascular disease, type I diabetes and sudden death syndrome in children evenly. The researchers suggested that the breeding programs should be designed to reduce the frequency of allele A1 (12).

It is reported that A2 allele increases breeding values for milk and protein production and reduces fat content in milk. Therefore the preference of A2 allele increased breeding value of protein production and reduces the frequency of A1 allele as a risk factor for human health (13). It was also indicated that diabetes mellitus is common in 1-14 years of old children of the ten countries that consume native cows' milk (14). In an experiment conducted by Keating *et al.* (15) an AS-PCR was evaluated to distinguish between the β -casomorphin-releasing variant and the non-releasing variant. The AS-PCR successfully distinguished β -casein variants in 41 of 42 animals as confirmed by sequence analysis.

Iranian Sistani cattle are a heavy built breed and used as dual-purpose cattle breed in Eastern Iran. This black-in-color breed, that is also native to Pakistan and Afghanistan, is a genetic resource that shows special features of adaptation to rustic environments. Such characteristic has become a biotype of great interest for the meat production industry within the last few years (16). Mazandarani cattle is a humpback breed native to Caspian Sea shores in north of Iran. Also, Taleshi is native of north of Iran and is a dual purpose (dairy - meat) cattle. Karimi *et al.* (17) estimated the effective population size, genomic inbreeding coefficients, autozygosity derived from runs of homozygosity, and genetic diversity in Iranian native cattle of Sistani, Taleshi and Mazandarani using dense SNP markers. The estimated effective population size (N_e) was relatively small for Sistani and Taleshi ($N_e=17$ and 22) and $N_e=107$ for Mazandarani breed.

The inbreeding coefficients estimated by runs of homozygosity varied between breeds. Sistani breed had higher proportion of long runs of homozygosity, likely to reflect recent inbreeding. Results also showed Sistani and Taleshi breeds are exposed to serious risk of extinction. Higher priority should be given to conservation

programming in Sistani breeds due to unique genetic composition and critical conservation status.

In the present study the genetic behavior of β -casein candidate gene was evaluated in three Iranian native breeds of Mazandarani, Taleshi and Sistani and also commercial breeds of Holstein and Simmental cattle to screening A1 and A2 alleles of β -casein gene using AS-PCR and melting curve analysis.

Materials and Methods

DNA amplification and Genotyping

Blood samples were collected from Holstein (N=119), Simmental (N=32), Mazandarani (N=103), Taleshi (N=81) and Sistani (N=59), cattle in EDTA treated tubes as an anticoagulant. The samples were kept in a cooling chain, transferred to the laboratory and stored at -20 °C until further analysis. Genomic DNA was extracted using modified salting out method (15). The AS-primer pairs of F-5'- GCCCAGATGAGA-GAAGTGAGG -3', R1 (allele A1) - 5'- GATGTTTTG-TGGGAGGCTGTTAT -3' and R2 (allele A2) - 5'- GATGTTTTGTGGGAGGCTGTTAG -3' (12) were used to amplification of a 854 bp fragment from intron 6 and exon 7 of bovine β -casein gene in PCR reactions with a total volume of 25 μ l, containing 2.5 μ l 10x PCR buffer, 0.2 mM dNTP-mix, 2.5 mM MgCl₂, 100 ng DNA and 1 units *Taq* DNA polymerase. The thermo-cycling conditions included an initial denaturation at 95 °C for 10 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 58 °C for annealing temperature and 1 min at 72 °C. A final elongation step was carried out at 72 °C for 10 min. for each samples, two tubes were used to amplify A1 and A2 alleles. Genotyping of individuals was done after ethidium bromide staining of PCR product and direct visualization of bands on agarose gel after electrophoresis. Amplification of two reverse primers shows the heterozygous, R1 showed A1A1 homozygous and R2 shows A2A2 homozygous individuals (15, 18).

Melting curve analysis

To ensure the efficiency of PCR-based method for identifying different alleles in the studied cattle, the melting curve analysis of the PCR products were used. Since the 854 bp fragment was not suitable for melting analysis, so the primer pairs of F: 5'-CAGTCTCTAGTC-TATCCCTTCC-3' and R: 5'-CCTCCTTCACTTTG-GAGACT-3' was designed to amplify a small fragment contains 136 bp around the interested SNP of β -casein gene. For this purpose, a real time-PCR method with Rotor-gene 3000 RG-2072D instrument was used. The amplified samples were melted with a gradient from 65 to 95 °C with adding 0.5 °C per cycle and lagging for 25 second (95 °C was determined as optimum temperature for melting curve analysis). Different genotypes were determined by plotting melting temperature against fluorescent derived/melting derived (dF/dT). The population genetics parameters and Hardy-Weinberg equilibrium test were carried out using POPGENE software (19). The Fisher exact and chi-square test were performed with SAS software (version 9.1), to compare allelic and genotypic frequencies between different breeds.

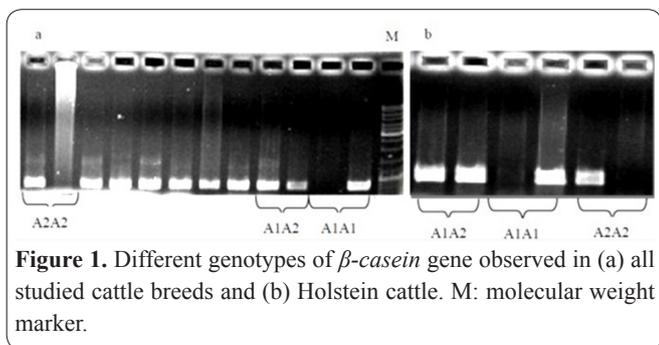


Figure 1. Different genotypes of β -casein gene observed in (a) all studied cattle breeds and (b) Holstein cattle. M: molecular weight marker.

Results

In this study an 854 bp fragment of β -casein gene was amplified from Holstein, Simmental, Sistani, Taleshi and Mazandarani cattle breeds. The AS-PCR generated three genotypes of A1A1, A1A2 and A2A2 with different frequencies across the studied breeds (Figure 1). The melting curve analysis confirmed the accuracy of allele specific PCR to distinguish between different genotypes (Figures 2 & 3). Results of genotyping and HW test for Sistani cattle are shown in Table 1. The genotype and allelic frequencies of 74.5, 16.9, 8.6 and 54.5 and 45.5% were observed for A1A2, A1A1, A2A2 and A1 and A2, respectively, and did not follow HW equilibrium ($P < 0.05$). In contrast, the A1 allele frequency (49.4) in Taleshi breed was lower than Sistani (54.5) breed. As shown in Table 2, three genotypes of A1A2 (61.37%), A2A2 (19.75%) and A1A1 (18.5%) were observed in Taleshi breed. The frequency of heterozygote genotype in Taleshi breed was lower than other native breeds of Sistani and Mazandarani. A relatively high frequency of favorable allele A2 (50.6) was observed in forest cows of Taleshi breed. This may indicate the desirability of Taleshi milk compared to Sistani. The Results of genotyping for Mazandarani cattle showed three genotypes of A1A2, A1A1 and A2A2 with the frequency of 73.8, 9.7 and 16.5%, respectively (Table 5). The frequency of heterozygosity in Mazandarani cattle was higher than two other native breeds. The high observed allele frequency of the desired A2 allele (53.4%) in Mazandarani cows was notable. The β -casein gene locus showed significant departure from HW-equilibrium in Taleshi and Mazandarani breeds ($P < 0.05$). As shown in Table 1, three genotypes of A1A2, A1A1 and A2A2 were found in Holstein population with the frequencies of 83.2, 8.4 and 8.4%, respectively. The frequency of heterozygous genotype was highest (65.62%) in Simmental cattle population. According to the chi-square test (χ^2), the Holstein and Simmental populations were not in equilibrium at β -casein gene locus (Table 1). The allele and genotype frequencies comparison between

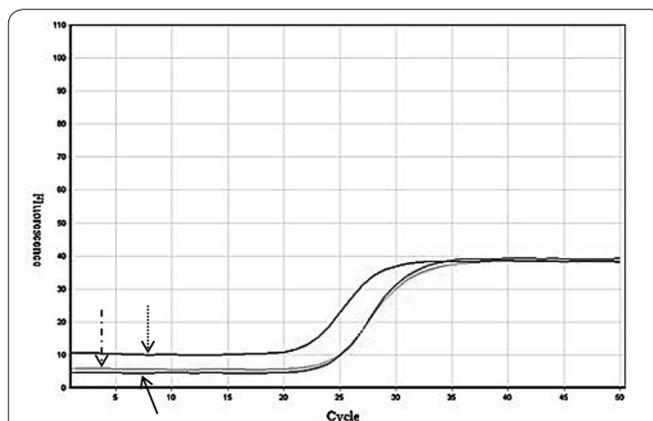


Figure 2. Amplifying of 136 bp fragment of β -casein gene by Real-time PCR. Solid, dash-dot and dot arrows showed TT (A1A1), CC (22) and CT (A1A2) genotype, respectively.

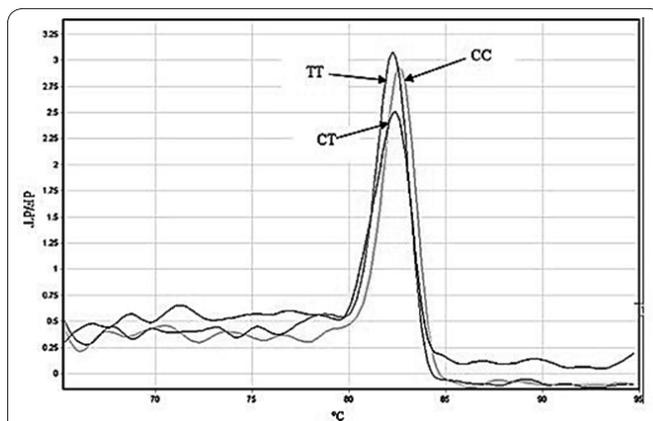


Figure 3. Plotting dF/dT versus melting temperature successfully distinguished three genotypes of CC, CT and TT that are corresponding to A2A2, A1A2 and A1A1, respectively.

studied breeds were done using Fisher exact test and the results are shown in Table 2. Except significant differences ($P < 0.05$) between Taleshi and Holstein breeds (Table 2), there were no significant differences in allele and genotype frequency between the other breeds (Table 2 & 3).

Discussion

In recent years, many researchers interested in research on the identification of the active components found in milk and dairy products and describe the method by which the mammalian physiology is adjusted by these components. Not surprisingly, a significant part of the investigations have been done to determine the strength of cow milk, dairy products or seek to influence some of the most important components of milk with physiological functions such as blood pressure (20-22) and immune system (23-25). For example, there are a

Table 1. Genotype and allelic frequencies and χ^2 test results for β -casein gene in studied breeds.

Breed	Genotypic frequency (%)			Allelic frequency (%)		χ^2
	A1A1	A1A2	A2A2	A1	A2	
Mazandarani	9.7	73.8	16.5	46.6	53.4	24.57
Sistani	16.9	74.5	8.6	54.5	45.5	19.72
Taleshi	18.5	61.37	19.75	49.4	50.6	5.61
Holstein	8.4	83.2	8.4	50	50	44.08
Simmental	18.75	65.62	15.63	51.57	48.43	6.57

Table 2. Comparison of allelic frequencies of β -casein locus between studied cattle breeds.

Breed	Number of animals	Allele frequency				Breed p-value			
		A1	A2	Sistani	Taleshi	Mazandarani	Holstein	Simmental	
Sistani	59	54.5	45.5	---	0.15	0.18	0.23	0.54	
Taleshi	81	49.4	50.6	0.15	---	0.14	0.003	0.88	
Mazandarani	103	46.6	53.4	0.18	0.14	---	0.16	0.38	
Holstein	143	50	50	0.23	0.003	0.16	---	0.89	
Simmental	32	51.17	48.43	0.54	0.88	0.38	0.89	---	

Table 3. Comparison of Genotypic frequencies of β -casein locus between studied cattle breed.

Breed	Number of animals	Genotypic frequency			Breed p-value				
		A1A1	A1A2	A2A2	Sistani	Taleshi	Mazandarani	Holstein	Simmental
Sistani	59	16.9	74.5	8.6	---	0.063	0.08	0.11	0.16
Taleshi	81	18.5	61.37	19.75	0.063	---	0.12	0.07	0.16
Mazandarani	103	9.7	73.8	16.5	0.08	0.12	---	0.09	0.15
Holstein	143	8.4	83.2	8.4	0.11	0.07	0.09	---	0.15
Simmental	32	18.75	65.62	15.63	0.16	0.16	0.15	0.15	---

considerable amount of evidence shows that main components in cow's milk, as well as various combinations or even yoghurt and cheese, can be set the blood pressure in human (25). Current knowledge in this area is rapidly expanding. In addition, special attention should be to the bioactive peptides derived from milk. Evidence is related the autism and diabetes shows that the amount of β -casein A1 anywhere in the world is high; there is a high rate of type 1 diabetes. The type 1 diabetes is an autoimmune disease, meaning the body attacks its own event. The cow A2 variant is natural and genuine, and due to a mutation, the A1 allele is created. The combination of A1 and A2 cattle are living in herds, so it is possible to rear A2 cattle. Goat's, sheep's and human milk are A2.

There is very clear evidence related β -casomorphine-7 to heart disease. Countries with high rates of heart disease are the countries that have high levels of β -casein A1 frequency. When two groups of rabbits fed with A2 and A1 milk, the A1 group has been suffering from arterial plaque (26). Adverse effects of β -casein A1 protein are one of the controversial ideas in dairy science (14, 27-30). Elimination of A1 allele by selecting the herd may be needs to identify the A1 allele effects on the properties of the milk. The A1 allele frequencies in different breeds altered between 0.01-0.06 (Guernsey), 0.09-0.22 (Jersey), 0.31-0.66 (Holstein), 0.43-0.72 (Ayrshire) and 0.71 (Red Danish) (2), clearly shows the positive effect of the A2 allele on reform the programs of dairy cows.

In this study we examine the β -casein gene polymorphism in Iranian Holstein, Simmental and native cattle of Sistani, Mazandarani, and Taleshi breed. In all studied breeds, the heterozygous of A1A2 genotype showed highest frequency ranged from 61.37 (Taleshi) to 83.2 (Holstein). The A2 allele frequency in Mazandarani and Taleshi breeds was higher than other breeds. Truswell

(31) showed that African and Asian cows are all A2, but in our study the A1 mutation was found in Iranian native cattle. The findings of the present study were in concordance with those reported for Pinzgau and Slovak Pinzgau breeds. Miluchova *et al.* (26) determined three genotypes of β -casein – homozygote genotype A1A1, heterozygote genotype A1A2 and homozygote genotype A2A2 with frequencies of 0.1261, 0.3333 and 0.5405 in Simmental breed; 0.1379, 0.4598 and 0.4023 in Holstein breed, 0.3034, 0.5168 and 0.1798 in Pinzgau breed. In population of Simmental and Holstein breeds the frequency of allele A2 (0.7072 and 0.6322) and in population of Pinzgau breed the frequency of the allele A1 (0.5618) were highest. Also, Miluchova *et al.* (32) reported the frequency of 0.56 for A1 allele in Slovak Pinzgau breed.

Our findings were not in agreement with results of Olenski *et al.* (33) that reported the allele frequency of 33% for β -casein A1 in Holstein bulls, Keating *et al.* (15) that reported the highest prevalence of A1 in dairy (77%), dual purpose (69%), and beef (50%) cattle breeds. Another study was undertaken to explore A1/A2 polymorphism in crossbred, Frieswal (HF X Sahiwal; N=172) cattle population using allele-specific PCR. All the three possible genotypes (A1A1, A1A2, and A2A2) were identified in Frieswal population. Overall frequency of A1 allele in the population observed to be 0.35 (34). Rahimi *et al.* (35) determined the frequency of β -casein variants in native cattle of Kermanshah Province in west of Iran by AS-PCR and showed that frequency of A1A1, A1A2, and A2A2 genotypes were 16.8, 42.7, and 40.5%, respectively. In that study a relatively high frequency of 38.2% was found for beta-casein A1 allele.

Also, results of the present study to be somewhat compatible with Haunsova *et al.* (36) that showed the

frequency of 54 and 46% for allele A1 and A2 in Slovakia Holstein cows and 60 and 40% in Slovakia Holstein bulls, respectively, as well as Kaminsky *et al.* (5) that reported frequencies of A1 (60%) and (A2) 40% alleles in Poland Holstein bulls. Kwai-Hang *et al.* (37) reported that the β -casein A1 did not exist in the Asian and African purebred cows. Mir *et al.* (38) determined the allele and genotype frequencies of genetic variants in β -casein gene and estimate the effect of these variants on milk yield in Sahiwal cattle. Genotypes were detected using SNaPshot genotyping method. The β -casein exhibited polymorphism with high allele frequencies of 0.93 for beta casein A2 allele. In that study, β -casein reported variant had no effect ($p > 0.05$) on 1st lactation and 2nd lactation milk yield. Also Ganguly *et al.* (39) reported the frequencies of 0.06, 0.32 and 0.44 for A1 allele in Ongole, Frieswal heifer and Frieswal bull, respectively.

The A1 allele was found in Iranian indigenous cattle with relatively high frequencies (Table 1). The β -casein gene locus showed no HW equilibrium in all studied breeds that it can be due to the small population size in the present study. Due to the adverse effects of β -casein A1 protein, the consumers want to reduce or eliminate this allele from their diet. Furthermore, the results of Friesian Holstein dairy cow in Germany showed positive effect of A2 allele on increasing protein and milk, whereas, A1 allele showed the opposite effect (40-41). Winkelman and Wickham, (42) and Ikonen *et al.* (43) reported the similar effect of A2A2 genotype on milk from Friesian and Ayrshire cows, respectively. Therefore, increase the frequency of A2 allele is offered. The effect of A2 allele was economically studied by Kearney *et al.* (44). The advantages of artificial insemination bulls with genotype A2A2 is dependent on the A2 allele frequency in bulls and cows in the time periods. The authors concluded that the benefits of a cow with A2A2 genotype are more than a cow with two other genotypes (assuming lactation 4000 kg/year). Assuming that content of β -casomorphine-7 of β -casein A1 milk is four times higher than the A2 milk, so that, A2 milk should get "upgrade-Health" property and find a new position for itself in the dairy industry market.

In conclusion, a relatively high frequency of β -casein A1 allele was observed in Iranian native cattle. Therefore, determine the genotypes and preference alleles A2 in these native and commercial cattle is recommended that may lead to two advantages of the increasing breeding value for protein function and reduce the protein A1, which is considered as a risk factor for human health.

A relatively high frequency of β -casein A1 allele was observed in Iranian native cattle. Therefore, determine the genotypes and preference alleles A2 in these native and commercial cattle are recommended.

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