

## Polymorphism of $\kappa$ -Casein Gene in Iranian Holsteins

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**Background:** Genetic polymorphism of milk proteins has been associated with composition, manufacture, and traits of milk. Caseins are the most important milk proteins that its genes are strongly linked and inherited together.  $\kappa$ -casein, which is a quantitatively minor constituent of bovine milk, is thought to play a critical role in organization, fixation and aggregation of casein micelles and firmness of curd during cheese making.

**Objectives:** In this study, we aimed to investigate the polymorphism of  $\kappa$ -casein gene in Iranian Holstein cows.

**Materials and Methods:** In this study,  $\kappa$ -casein gene polymorphism among 50 DNA samples of Iranian Holstein cows via polymerase chain reaction sequence analysis (PCR sequencing) was analyzed. For data analysis SPSS 11.5 (ANOVA test) was used.

**Results:** Four polymorphic sites that created four variants and seven different genotypes of  $\kappa$ -casein gene were identified. In this population the frequencies of A, B, C, and E alleles were as 0.391, 0.413, 0.087, and 0.109 respectively.

**Conclusions:** We suggest that the frequency of  $\kappa$ -casein gene B allele should be increased in Iranian Holsteins because it is an essential factor in marker-assisted selection for milk traits.

**Keywords:** Alleles; Caseins; Holstein Cows; Polymerase Chain Reaction; Sequence Analysis

### 1. Background

The most important milk proteins are caseins which are secreted from mammary gland cells. They constitute about 80% of bovine milk proteins and are divided into four principal classes:  $\alpha$ s1,  $\alpha$ s2,  $\beta$ , and  $\kappa$ -casein (1). About 95% of milk caseins are large colloidal particles known as micelles, on a dry matter basis. Casein micelles contain of 94% protein and 6% low weight molecular species referred to as colloidal calcium phosphate (2). These micelles increase the solubility of minerals and facilitate the transfer of nutrients from the mother to the offspring (3). Caseins and their genetic variants have been widely studied and reported as the essential factors associated with milk protein content and cheese yield (4-6).  $\kappa$ -casein, which consists about 15% of total milk caseins (2), has a crucial role in the formation, stabilization and aggregation of casein micelles thus effects the technological (7) and nutritional properties of milk (8, 9). The mature  $\kappa$ -casein protein has a peptide bond that is cleaved in the gut by the action of renin, to produce an insoluble peptide (para  $\kappa$ -casein or PKC) as well as a soluble hydrophilic

glycopeptide (caseinomacropptide or CMP) (10). The caseins are encoded by single copy genes clustered in a region about 200 kb on chromosome 6 in bovine (11-13), 4 in goat, human, and sheep, 5 in mouse, and 12 in rabbit (14); arranged in the following order,  $\alpha$ s1,  $\alpha$ s2,  $\beta$ , and  $\kappa$ -casein (15). The  $\kappa$ -casein gene comprehends a 13-kb sequence subdivided into five exons and four introns (16) and sequences of this gene constitute about 25% of the casein gene cluster (15).  $\kappa$ -casein gene 5' region is organized differently from that of other casein genes, showing that its expression is independent of other casein genes (16). Polymorphisms of milk proteins are important because genetic selection and genetic characterization of bovine breeds are related to promotion of positive properties of milk and cheese yield (17, 18). Different breeds of cattle have various allelic variants for the  $\kappa$ -casein gene, including A, B, C, E, F, G, H, I, and A1 (19, 20).

### 2. Objectives

In this study the polymorphism of  $\kappa$ -casein gene in Iranian Holstein dairy cows was investigated.

#### Implication for health policy/practice/research/medical education:

In the present study, we reported on mutations of the  $\kappa$ -casein gene in Iranian Holstein cows via PCR-sequencing. The B allele frequency of this gene was higher than the other ones. The use of this allele as a marker to assist selection of Holstein cows around the world will increase the milk protein and cheese yield therefore, we suggest using this allele to improve the milk quality.

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### 3. Materials and Methods

The animal sample consisted of 50 Holste in cattle randomly selected from the second parity cows in Ghiam Dairy Co. (Iran). Peripheral whole blood was collected from jugular veins into tubes containing citrate as an anticoagulant. High molecular weight DNA was extracted by a modified salting-out method (21). Primer pairs targeting all coding regions of the  $\kappa$ -casein gene were designed based on a reference GenBank sequence, NC\_007304, using the Vector NTI software v10.1 (Invitrogen). An annealing temperature of 60°C and concentrations of MgCl<sub>2</sub> (1–4 mM) was used to optimize the PCR; the reaction consisted of template DNA (50 ng), primers (16 pmol for each), dNTPs (0.2 mM), 1X buffer, and 1 U Taq polymerase in a 25  $\mu$ L reaction. The DNA was denatured at 95°C for 5 minutes. Reactions were cycled 30 times; 95°C 30 s<sup>-1</sup>, annealing temperature 60°C 30 s<sup>-1</sup>, extension 72°C 30 s<sup>-1</sup>, and finally incubated at 72°C for 5 minutes. All of the PCR products were electrophoresed at 150 V for 40 minutes through a 2% agarose gel containing 1X TBE buffer and 0.14 mg.mL<sup>-1</sup> ethidium bromide to check whether amplification had been successful. The purified PCR products were sequenced (Macrogen, Korea) in both directions using the appropriate PCR primers (22, 23).

F primer: 5'-CCCATTTCGCCTTCTCTGTA-3'

R primer: 5'-CAGCGCTGTGAGAAAGATGA-3'

#### 3.1. Statistical Analysis

Statistical analysis was done by the SPSS software (version 11.5) (Illinois, USA) and the comparison between frequency of genotypes and alleles was analyzed by ANOVA (post-hoc).

### 4. Results

#### 4.1. Observation of the PCR Products

Electrophoresis of PCR production and ethidium bromide staining showed that only one bond with 13kb weight for  $\kappa$ -casein gene existed in the study samples (Figure 1).

#### 4.2. Sequencing

Polymorphic sites in exon 4 and different genotypes in Holstein  $\kappa$ -casein gene were identified, as follows:

1- Sequencing analysis of 28 samples indicated that in the positions 2523, 10711, 10731, 10825, 10828, 10863, and 10884 of  $\kappa$ -casein gene, the G, G, T, C, C, A and A nucleotides were present and created the A variant. The frequency of this variant in this study was 0.391.

2- At positions 10828 and 10863 of the  $\kappa$ -casein gene, two mutations of C→T (Transition) and A→C (Transversion) occurred, resulted in an Isoleucine 136 instead of

Threonine 136 and an Alanine 148 instead of Aspartate 148 amino acids in the protein and created the B variant of  $\kappa$ -casein. Frequency of B allele in this population was estimated as 0.413 (P < 0.05).

3- At position 10711 of this gene, G→A (Transition) mutation was present, which resulted in a Histidine 97 instead of Arginine 97 amino acid. This mutation created a C variant. Also in this variant, two previous mutations were present. Frequency of C allele was estimated as 0.087 (P < 0.05).

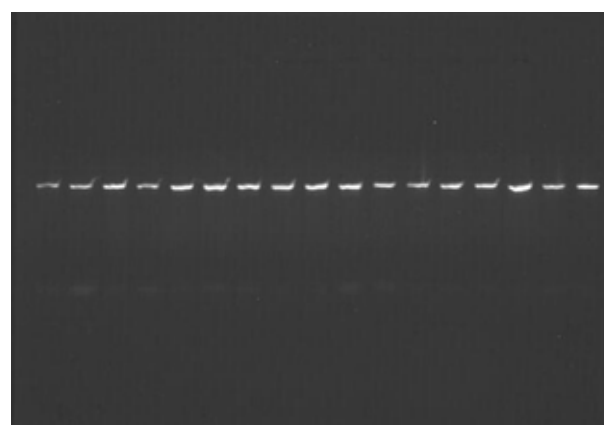
4- At position 10884 of  $\kappa$ -casein gene A→G (Transition) mutation occurred, which resulted in Glycine 155 instead of Serine 155 amino acid and created the E variant of this gene. Frequency of the E allele was estimated as 0.109 (P < 0.05) (Table 1).

Frequencies of seven different genotypes and four different alleles of  $\kappa$ -casein gene in Holstein cows are summarized in Tables 2 and 3. The A and B alleles frequencies in these breed and other breeds are summarized in Table 4.

### 5. Discussion

Cattle breeds' allelic variants have demonstrated that the B allele of  $\kappa$ -casein may allow improvement in milk quality for manufacturing processes, being excellent for cheese making due to faster coagulation and firmer curd (26, 27), unlike the E variant of  $\kappa$ -casein which has been found to be related to an inferior coagulation feature (28, 29). In this study, four polymorphic sites in exon 4 and 7 of different genotypes of Holstein  $\kappa$ -casein were identified and the B allele frequency was higher (0.413) compared to the other alleles (A, C, and E). Genetic improvement of breeds or herds could be associated with the B allele and therefore we should try to select cows with this  $\kappa$ -casein allele for production purposes.

Figure 1. Bond of PCR Product on Electrophoresis Gel



The pointer show  $\kappa$ -casein gene bond (13 kb).

**Table 1.**  $\kappa$ -casein Gene Mutations in Iranian Holsteins

Amino Acids Exchange	Mutations Type	Nucleotide Position	Polymorphic Region	Variant
<b>Arg→His</b> CGT→CAT	G→A (Transition)	10711	+290	C
<b>Thr→Ile</b> ACC→ATC	C→T (Transition)	10828	+407	B
<b>Asp→Ala</b> GAT→GCT	C→A (Transversion)	10863	+443	B
<b>Ser→Gly</b> AGT→GGT	A→G (Transition)	10884	+464	E

**Table 2.** Genotype Frequencies of  $\kappa$ -casein Gene in Iranian Holsteins

Genotype	Genotype Frequency
AA	0.174
AB	0.304
AC	0.087
AE	0.043
BB	0.261
CC	0.043
EE	0.087

**Table 3.** Allele Frequencies of  $\kappa$ -Casein Gene in Iranian Holsteins.

Allele	Allele Frequency
A	0.391
B	0.413
C	0.086
E	0.108

**Table 4.** The frequencies of A and B alleles in different Iranian Cattle Breeds

Breed	A Allele of $\kappa$ -Casein	B Allele of $\kappa$ -Casein	Reference
<b>Iranian Holstein</b>	0.391	0.413	Present study
<b>Crioulo da Argentina</b>	0.647	0.353	(24)
<b>Argentine Holstein</b>	0.656	0.344	(24)
<b>Gyr</b>	0.930	0.070	(18)
<b>Finnish Ayrshire</b>	0.612	-	(29)
<b>Nelore</b>	0.910	0.090	(18)

A study of alleles frequencies of caseins in the Finnish Ayrish breed showed that A and E allele frequencies of  $\kappa$ -casein were 0.612 and 0.307, respectively (29). In another study, allele frequencies of  $\kappa$ -casein gene were examined in 1316 cattles from the Brazilian Bos Indicus breed and the highest frequency belonged to the B allele, which was 0.30 (25). On the other hand, in different breeds, the frequency of this allele ranged from 0.01 to 0.18 (30). Due to a single base mutation in the  $\kappa$ -casein locus of the B variant, Isoleucine substituted by Threonine and Aspartic acid replaced by Alanine (31) were found to be related to different sizes of micelles, thermal resistance, shorter coagulation, and better curdles, which are important for cheese making and cheese yield (32, 33). In a study in China, the A allele of  $\kappa$ -casein gene was dominant in Chinese Holstein cattle and its frequency was estimated as 0.73 (34), which is similar to the results of a study on Indian goat (35) and Russian cattle breeds (36). In the casein molecule, variants that are named post-transcriptional variants are also detected (18, 37). Polymorphisms of  $\kappa$ -casein gene have been reported in different cattle breeds and

the frequencies of all alleles of this gene have been estimated (17, 18, 38). In view of the existing evidence on the whole casein group, the casein haplotype effects on productive traits have been examined and confirmed. Moreover, noncoding sequence mutations could affect specific protein expression, milk composition, and cheese making. Milk protein variants are also useful tools for breed characterization, variety, and phylogenetic researches. Improvement of human nutrition quality is dependent on beneficial allele selection in animals for milk proteins and should be tested to produce the specific milk, e.g. hypoallergenic milk (39). Recently, researchers found 16 polymorphic sites at the  $\kappa$ -casein (CSN3) gene in domesticated dairy goat (*Capra hircus*). Thirteen mutation sites created protein variants and amongst, three were silent mutations in exon 4 (40). In a sample of 540 dairy goats, 67 different haplotypes with frequency of 0.01 and 27 with frequency of 0.03 were reported. Analysis of 41 White Shorthaired (WSH) trio families and 44 Brown Shorthaired (BSH) trio families in two dairy goat breeds showed that respectively 14 and 20 haplotypes were pres-

ent. Various genomic techniques were used to type the casein loci. Twenty-two different combinations of  $\kappa$ -casein alleles were found (41). Study of casein complex by milk isoelectrofocusing and analysis at the DNA level in three goat breeds from Northern Italy showed that the majority of all known polymorphisms were present and a new allele of  $\beta$ -casein was identified, which seemed to be specific to the Frisa breed. It was named  $\beta$ -casein\*E and characterized by a transversion mutation (TCT→TAT) responsible for an amino acid replacement (Ser166→Tyr166) in this protein (24). In Iran, there has been no selection for specific protein variants in breeding programs and through the current study the allele frequency of  $\kappa$ -casein gene was determined, probably being helpful for selection of cow breeds.

Polymorphisms of  $\kappa$ -casein gene have not been previously reported for Holstein via the PCR-sequencing method. Genetic variants detection by means of PCR-RFLP method was limited to mutation identification in the gene and we suggest the PCR-sequencing method for discovering new  $\kappa$ -casein gene mutations. The polymorphism of milk proteins affects the milk composition and cheese quality and the mutations should be used for molecular marker-assisted selection. In this study, four polymorphic sites in exons 4 and 7 in different genotypes of Holstein  $\kappa$ -casein were identified and the B allele frequency was higher (0.413) compared to the other alleles (A, C, and E).

In the present study, we reported mutations of  $\kappa$ -casein gene in Iranian Holstein cows using the PCR-sequencing. The B allele frequency of this gene was higher than the other alleles. The use of this allele as a genetic marker in Holstein cows around the world may increase milk protein and cheese yield, hence we suggest using this allele to improve milk quality.

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## Author's contribution

Homayon Reza Shahbazkia suggested the primary idea and the protocol, abstracted the research, analyzed the data, and wrote the manuscript. Zahra Molavi Choobini recorded samples, designed and did the laboratory research, analyzed the data, wrote the manuscript and carried out its submission. Mohammad Shadkhast, Hamdollah Moshtaghi and Said Habibian Dehkordi supervised the laboratory research.

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## Financial Disclosure

We declared no conflict of interest in this study.

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