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Analysis of bovine beta-casein A1 and A2 allele frequency in Holstein-Friesian cows by Real-time PCR with fluorescent hybridization probes

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ABSTRACT

A2 milk popularity is increasing across the world and novel molecular techniques have been evaluated to develop reliable methods. This study aimed to genotype Holstein-Friesian cows concerning their A1/A2 status using Real-time PCR assay with the specifically designed FRET hybridization probes. In this context, DNA samples were obtained from 310 Holstein-Friesian milk samples. Concerning the Real-time PCR assay, the melting temperature of each amplicon was analyzed and the melting data was converted to a derivative plot using the LightCycler 480 System. The sensor probe was designed to match the wild-type sequence in the target DNA. In the Real-time PCR assay, the melting peaks obtained in the Real-time PCR assay were highly decisive and consistent for each genotype regarding CCT→CAT alteration. The results indicated a remarkably high frequency of the A2 allele (68%) and a considerable frequency of heterozygous animals (0.41). Population genetic analysis showed intermediate levels of genetic variability and biodiversity. The A2-herd conversion process is a complex process consisting of genetic testing of both cows and calves, evaluating replacement rates, and the conversion of heterozygotes by using A2-genotyped bull semen. In this sense, the key point is a reliable and rapid genotyping method to produce A1-free milk. This study suggests that Real-time PCR assay with the specifically designed FRET hybridization probes is a preferable method for A2 genotyping, and may be useful for further studies and instructive for companies or breeders who aim to produce A2 milk.

Key words: cattle; A2 milk; *β*-casein; genetic testing; FRET hybridization probes

Introduction

In dairy cattle, milk yield and composition are typical polygenic traits. Phenotypes reflect the joint action of large numbers of polygenes or quantitative trait loci (QTL) with the contribution of environmental effects (GEORGES et al., 1995; ARDICLI et al., 2018). In this context, bovine chromosome 6 (BTA6) is one of the most remarkable interests for many research groups in searching for efficient QTL concerning milk production traits (OLEŃSKI et al., 2012). Previous studies have shown that this genomic region harbors many QTLs which are responsible for milk yield and protein or fat content (KHATKAR et al., 2004; SCHOPEN et al., 2009). In addition, SCHOPEN

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et al. (2009) reported that a part of the phenotypic variance (~3.5%) for milk protein content may be associated with genomic alterations within the beta-casein gene, also described as *CSN2*.

The beta-casein, which is fairly important for the casein micelle structure, constitutes up to 45% of the casein of bovine milk (MİLUCHOVÁ et al., 2013). This gene is the most polymorphic milk protein gene, with 13 protein variants including A, B, C (ASCHAFFENBURG, 1961), A1, A2, A3 (KIDDY et al., 1966), D (ASCHAFFENBURG et al., 1968), E (VOGLINO, 1972), F (VISSER et al., 1991), X (VISSER et al., 1995), G (FORMAGGIONI et al., 1999), H (HAN et al., 2000), I (JANN et al., 2002). The most common forms of beta-casein in dairy cattle breeds are A1 and A2, while B is less common, and A3 and C are rare (FARRELL et al., 2004; KEATING et al., 2008).

In bovine milk, the original beta-casein protein was A2. Alleles differ by point mutations that result in amino acid alterations. In this context, alleles A1 and A2 of the beta-casein locus differ in amino acid position 67, where A1 encodes histidine and A2 encodes proline, utilizing codons CCT → CAT (OLEŃSKI et al., 2012; MILUCHOVÁ et al., 2013). It is worth noting that the digestion of A1 beta-casein can result in the production of bioactive beta casomorphin-7 (BCM-7), which is associated with unfavorable effects on components of the vascular and immune systems. Accordingly, consumption of A1 milk has been reported to be a risk factor for many diseases and syndromes, such as coronary heart disease, diabetes mellitus (type I), autism, and even schizophrenia (TRUSWELL, 2005).

Apart from the studies on human health, the same substitution (A1/A2) has been reported to increase milk protein yield and content, and to decrease fat yield and content (IKONEN et al., 2001; OLEŃSKI et al., 2012). However, results from previous studies are often conflicting. Still, there is the question of whether the published reports were sufficiently satisfactory to draw reliable conclusions. If the above-mentioned interpretations about the association between beta-casein A1/A2 and the incidence of diseases are strongly confirmed, focusing on this genomic region would increase its importance, and furthermore, the results may influence not only milk production but also human health.

In comparison to conventional detection methods, real-time probe-based PCR assays have been shown to be preferred molecular techniques with increased sensitivity and specificity (LASSAUNIERE et al., 2010). The fluorescence resonance energy transfer (FRET) technology is a nonradiative form depending on long-range dipole-dipole interactions, in which process a photoexcited donor transfers energy to a proximal acceptor in the ground state (QUAN et al., 2020). In this technique, the acceptor must absorb energy at the emission wavelength of the donor. Real-time PCR detection by the conventional fluorescence melting curves is a common, simple, and rapid method, but the primer dimers may complicate the interpretations, and even cause unreliable results concerning the melting curves (CALERO et al., 2009). Nowadays cattle are genotyped for many different purposes such as selection for improving milk production and constituents. On the other hand, various genotyping methods are also used for some more specific targets, such as obtaining A2 milk. In this sense, the main objective is to obtain pure A2 milk and there is no room for error in genotyping. Real-time PCR assays with specifically designed hybridization probes are confidential and very effective methods, although they seem to be costly molecular techniques. Taken together, the aim of this study was to determine the genetic variability of the beta-casein gene in Holstein-Friesian cows using a rapid and convenient Real-time PCR assay with the specifically designed FRET hybridization probes.

Materials and methods

Animals. A total of 310 Holstein-Friesian cattle were analyzed. The studied population consisted predominantly of first lactation cows. They were raised on the same commercial farm located in the southern Marmara region of Turkey. The cows were housed in free-stall barns with the same management procedures and fed the same diets according to standard commercial practices.

DNA extraction and genotyping. Genomic DNA was isolated from milk samples using the High Pure

PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). The concentration of DNA was obtained according to optical density ratio (OD) at 260/280 wavelength to document quantity (ng/μL) and purity of extraction using a spectrophotometer (NanoDrop 2000c, Thermo Scientific, Wilmington, DE, USA).

Custom primers were chosen using the National Center for Biotechnology Information (NCBI) database. FRET probes were evaluated using the Light Cycler Probe Design Software 2.00 (v.1.0). The assay including primer and probe sequences was as follows:

Sense primer:
CAGTCTCTAGTCTATCCCTTCCCT

Antisense primer:
AGCCATAGCCTCCTTCACTT

Donor Probe:
CATCCCTAACAGCCTCCCACA-FL

Acceptor Probe: LCRed640-ACATCCCTCC
TCTTACTCAAACCCCTGTG-pho

Primer-BLAST was used to ensure that the primer sets amplified DNA products with high specificity, and thus, to avoid non-specific amplification. The real-time PCR reaction was performed using a LightCycler® 480 (Roche Diagnostics). Thermocycling was carried out in a final volume of 10 μL, containing 2.5 μL of sample DNA, 0.5 μL of each primer, 0.15 μL of each probe (FL and LC probes), 1 μL of Mg^{+2} (25 mM), 4.2 μL of molecular biology grade H_2O (nucleic acid and nuclease-free), and 1 μL of Fast Start Taq DNA polymerase (Roche Diagnostics).

After a 10 min initial denaturation at 95˚C, the cycling protocol consisted of 50 cycles of denaturation at 95˚C for 5 s, annealing at 55˚C for 10 s, and elongation at 72˚C for 15 s. This was followed by one cycle of 95 °C for 15 s and 40 °C for 45 s to generate an amplicon-specific melting curve. LightCycler® 480 Gene Scanning software was used to analyze the melting temperature (Tm Calling) of each amplicon and to convert the melting data to a derivative plot. Individual analyses of the samples were repeated in duplicate.

Evaluation of the genotypic data. Allele frequency and polymorphism under the Hardy-Weinberg equilibrium (HWE) were analyzed using the Cervus software v3.0 package. Population genetic indices, including heterozygosity (He), homozygosity (Ho), effective allele numbers (Ne), and the polymorphism information content (PIC), were calculated according to the method given by NEI and ROYCHOUDHURY (1974) and BOTSTEIN et al. (1980). The Fixation index (F_{IS}) was estimated from the values of theoretical (H_{the}) and experimental (H_{exp}) heterozygosities, and the level of possible variability realization (*V*%) was estimated on the basis of the suggestions by CROW and KIMURA (1970). The Shannon-Weaver diversity index (*H´*) was calculated according to SHANNON and WEAVER (1948). Moreover, the Simpson index (*D*) was estimated as previously described by SIMPSON (1949).

Results

Assay assessment. The melting peaks obtained in the Real-time PCR assay were highly decisive and consistent indicators for each genotype (Fig. 1). The sensor probe was designed to match the wild-type sequence (A2) in the target DNA. This resulted in a higher temperature (Tm: ~65°C). A mismatched mutant sequence (A1) in the target DNA provided less stability to the wild-type-matched sensor, which melted away at a remarkably lower melting temperature (Tm: ~57°C). In Fig. 2, melting profiles were represented, including curves (2A) and melting peaks (2B), for the detection of three genotypes of the *CSN2* gene identified with the FRET technology. As shown in Fig. 2B, a heterozygote sample yielded a melting peak corresponding with both wild- and mutant types. Genotype output profiles for the subset of 310 Holstein DNAs, along with control samples, are presented in Fig. 3. Melting curves and the derivative melting peaks for each genotype were consistently distinguishable. The assay profile concerning individual genotype peaks revealed 147 samples corresponding to wild type A2 allele (nucleotide: CC), 127 to heterozygote A1A2 (nucleotide: AC), and 36 mutant A1 alleles (nucleotide: AA).

Genetic variability. The genotype and allele frequencies, population genetic indices, and compatibility with the HWE are presented

in Table 1. Among the 310 Holstein-Friesian heifers included in this study, all three genotypes were detected: homozygote genotype A1A1 (36 animals), heterozygote genotype A1A2 (127 animals), and homozygote genotype A2A2 (147 animals). In the total population of cattle, even though the A2A2 genotype frequency was close to the heterozygote genotype frequency (0.41), homozygotes A2A2 (0.47) were the most frequent, while the A1A1homozygotes (0.12) were the least frequent. This indicates the superiority of allele A2 (0.68). Moreover, satisfying results were obtained for population genetic variability indices. In this respect, Ne was found to be 1.77 and the PIC was 0.34 which indicates a mildly informative genetic marker regarding the studied Holstein population. Genotype frequencies were examined for compatibility with the HWE, and the results indicated that no deviation was observed in the population analyzed. The diversity indices (*H′* and *D*) suggested admissible biodiversity (Table 1).

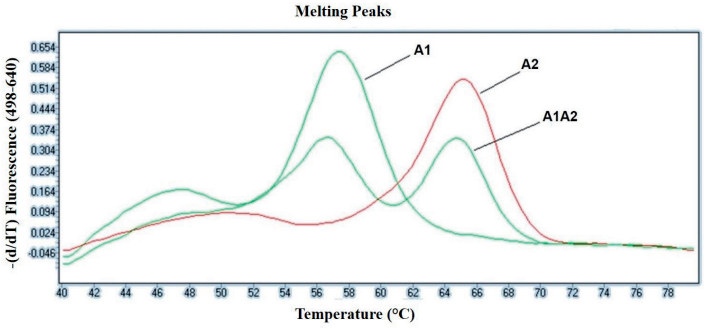

Fig. 1. Melting curve analysis illustrating the detection of three genotypes of the *CSN2* gene identified with the specific fluorescence resonance energy transfer (FRET) hybridization probes.

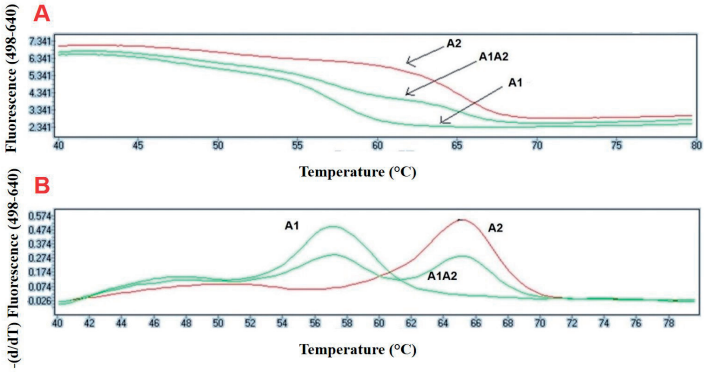

Fig. 2. Melting curves (A) and melting peaks (B) for the detection of three genotypes of the *CSN2* gene identified with the specific fluorescence resonance energy transfer (FRET) hybridization probes.

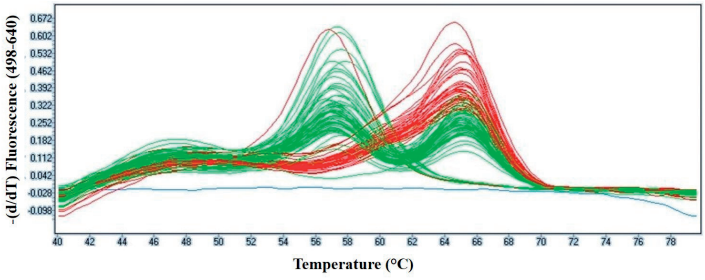

Fig. 3. Melting peaks for a batch of cattle DNA samples in the Real-time assay.

Table 1. Genotype and allele frequencies, population genetic parameters, the biodiversity indices, and compatibility with the Hardy–Weinberg equilibrium in the bovine beta-casein gene concerning A1/A2 alleles.

Locus	*CSN2*		
Genotypes	A1A1	A1A2	A2A2
n	36	127	147
GF	0.12	0.41	0.47
EGF	0.10	0.44	0.46
Alleles	A1	A2	
AF	0.32	0.68	
H_{the}	0.4352		
H_{exp}	0.4096		
Ho	0.5648		
F_{IS}	0.0579		
LPVR	0.6307		
Ne	1.7705		
PIC	0.3405		
H´	0.9694		
D	0.4043		
χ^2(HWE)	1.1214		
*P**	0.2896		

The *CSN2*: beta-casein; *n*: number of experimental cows; GF: genotype frequency; EGF: the expected genotype distribution according to HWE; AF: allele frequency; H_{the}: theoretical heterozygosity; H_{exp}: experimental heterozygosity; Ho: homozygosity; F_{IS}: fixation index; LPVR: level of possible variability realization; Ne: number of effective alleles; PIC: polymorphism information content; *H´*: the Shannon-Weaver diversity index; *D*: Simpson index; χ^2(HWE): Hardy–Weinberg equilibrium χ^2 value.
*P > 0.05 – consistent with HWE.

Discussion

Developing new molecular genetic techniques or reclaiming the conventional methods with some crucial modifications are highly valuable in A1/A2 identification. In this study, 310 Holstein-Friesian cows were genotyped using a Real-time PCR assay with the specifically designed FRET hybridization probes. Despite its high price, this technique can be recommended as a reliable and rapid method for discriminating the bovine beta-casein A1/A2 alleles. Genotype output profiles regarding melting curves and the derivative melting peaks for each genotype indicated that the technique can be considered a highly sensitive application for genotyping animals from milk samples. Although there are conventional methods that are inexpensive but with limited reliability such as the amplification created restriction site method followed by PCR assay (ACRS-PCR), and PCR-RFLP, novel approaches to genotyping, for instance, the hydrolysis probes system (TaqMan) (MANGA and DVOŘÁK, 2010), rhAmp genotyping (GIGLIOTI et al., 2020), and the FRET hybridization probes, can provide higher accuracy which is crucial to provide commercialized A2 milk.

The bovine beta-casein gene encodes the structural milk protein which is incorporated into the

casein micelle formation (SOYUDAL et al., 2018). Apart from its potential effects on human health, beta-casein has been shown to be an important gene regarding milk yield and component traits. Mostly, the major impact reported is regulating milk fat and protein. However, the results from previously published papers are often conflicting. One of the possible reasons for this situation is the unbalanced genotypic distributions in the studied populations. Testing for departures from the assumption of the HWE has been widely recommended as a preliminary step in genetic analysis. Population substructure is the main cause of deviation from HWE. In this study, the genotypic distribution was under HWE in the Holstein-Friesian cows. On the other hand, the usefulness of a genetic marker in segregation analysis or the efficacy of the marker in selection programs is directly related to its population genetic parameter levels. The results of the present study showed that beta-casein can be evaluated as an intermediately informative and utilizable marker. In this context, the PIC was between 0.25 and 0.50; Ne was ~1.77 and the He was ~0.44. A similar interpretation can be accepted for the evaluation of biodiversity indices including the *H′* and *D* (Table 1). The results indicated a mild diversity level in the studied population.

Holstein-Friesians have the A1 and A2 variants at approximately equivalent allele frequency (or higher A2). Not surprisingly, the breed is an important factor for the A2 frequency. For instance, Guernsey cattle show a predominance of the A2, with a remarkable frequency of 96-98%, but this percentage may decrease to 50% in the Jersey breed (MENCARINI VOISIER, 2013). Here, it is important to note that the genotypic and allelic frequencies may differ in different populations of the same breed. From a broad perspective, the main application is to perform herd conversion to provide A2 animals. The primary sequence, consisting of histidine (A1) to proline (A2) alteration at position 67, is encoded by a pair of genes located on BTA6, which have a codominant and additive effect (MENCARINI VOISIER, 2013; SOYUDAL et al., 2018). Thus, there are three possibilities, including the homozygous A1A1 and A2A2, and the heterozygous A1A2 genotypes. The progeny resulting from homozygous A2 dams mated with homozygote A2 sires will invariably be A2A2. To perform a successful A2 herd conversion some key parameters should be considered, such as the initial allele frequencies, herd size, replacement rate, involuntary culling rate, and the age of the animals. Moreover, decision variables include yearlings to A2 semen and sex-selected semen (MENCARINI VOISIER, 2013). Heterozygotes can be reclaimed by using A2 genotyped semen (obtained from certified homozygous A2 bulls) to acquire A2 calves. In this multi-step selection process, choosing a useful and reliable genetic test is very important. This study suggests that the qPCR assays, improved by FRET hybridization probes, may be suitable for recent applications to provide commercialized A2 milk from A2-converted cow herds.

Consequently, the A2-herd conversion process is a complex process with the genetic testing of both cows and calves, together with artificial insemination. Adequate herd size is a prerequisite for high cow replacement rates. In this study, a herd of 310 Holstein-Friesian cows was genotyped using a Real-time PCR assay with the specifically designed FRET hybridization probes. The results revealed that 147 animals were homozygous for beta-casein A2. Here, it should be noted that the herd consisted of 36 A1A1 homozygotes to be culled, and 127 heterozygotes to be converted by using A2 bull semen, if a distinctive A2 selection will be performed. However, these decisions can be realized by confidential genetic testing. In conclusion, genotyping of dairy cattle using Real-time PCR assay with the FRET hybridization probes and primers presented in this study was evaluated as an effective method concerning A2 milk production.

Conflict of interest statements

The authors declare that they have no conflict of interest.

Acknowledgments

This study does not contain any invasive procedures in animals performed by any of the authors. Thus, no ethics statement was required for this work because the DNA was extracted from the milk samples.

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ARDICLI, S., H. SAMLI, F. BALCI: Analiza učestalosti goveđeg beta-kazeina tipa A1 i A2 u krava holštajnsko-frizijske pasmine PCR-om u stvarnom vremenu s fluorescentnim hibridizacijskim sondama. Vet. arhiv 93, 279-286 2023.

SAŽETAK

Popularnost mlijeka s A2 tipom beta-kazeina sve je veća diljem svijeta što je popraćeno razvojem novih i pouzdanijih metoda za molekularne analize. Cilj je ovog istraživanja bila genotipizacija holštajnsko-frizijskih krava s obzirom na A1/A2 status primjenom PCR-a u stvarnom vremenu (RT-PCR) s posebno dizajniranim FRET hibridizacijskim sondama. DNA je dobivena iz uzoraka mlijeka 310 krava. RT-PCR-om analizirana je temperatura taljenja svakog amplikona te su ti podaci uz upotrebu sustava LightCycler 480 korišteni za iscrtavanje grafičkih prikaza. Senzorska sonda dizajnirana je tako da odgovara divljem tipu sekvencije u ciljnoj DNA. Vršne vrijednosti taljenja dobivene RT-PCR-om bile su postojane razlučivosti za svaki genotip s obzirom na promjenu CCT→CAT. Rezultati su pokazali vrlo visoku učestalost alela A2 (68 %) i znakovitu učestalost heterozogotnih životinja (0,41). Genetska analiza u populaciji pokazala je srednje vrijednosti genetske varijabilnosti i bioraznolikosti. Proces usmjeravanja uzgoja prema životinjama s A2 tipom mlijeka složen je proces koji uključuje genetsko testiranje krava i teladi, procjenu stopa zamjene i konverziju heterozigota upotrebom A2-genotipiziranog sjemena bikova. U tom je smislu ključna brza i pouzdana metoda genotipizacije životinja koje proizvode mlijeko bez A1 tipa beta-kazeina. Ovo istraživanje pokazuje da se RT-PCR s posebno dizajniranim hibridizacijskim FRET sondama može preporučiti za A2 genotipizaciju goveda. Navedena metoda mogla bi biti korisna u daljnjim istraživanjima te u industriji i u uzgoju stada za proizvodnju mlijeka s A2 tipom beta-kazeina.

Ključne riječi: goveda; A2 mlijeko; *β*-kazein; genetsko testiranje; FRET hibridizacijske sonde