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A New Primer Designing for PCR-RFLP Analysis of A and B Genetic Variants of Bovine Kappa-Casein

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Abstract: Bovine milk contains about 3.4% high-quality protein and it is very essential for human nutrition. Kappa (κ) casein is one of the milk proteins and encoded by the *CSN3* gene. Studies have shown that κ-casein has an important influence on milk properties and the manufacturing of milk. κ-casein takes role as a stabilizing factor during the curdling of milk, which makes it desired in the cheese factory. A and B genetic variants of κ-casein are well-known and well-studied. For the genotyping of bovines, the PCR-RFLP approach has been used. For this aim, different primer pairs have been used to amplify the polymorphic region of the *CSN3* gene. In a previous study, the *Hinf*I enzyme digestion of the polymorphic region resulted in short and similar length fragments. In agarose gel electrophoresis, separation of very similar DNA fragments is almost impossible and interpretation of the short DNA fragment sometimes causes challenges. Therefore, in this study, a new primer design was described. Using the new primer, longer DNA fragment was amplified successfully and *Hinf*I digestion of the PCR product let to a longer and very different lengths of DNA fragments, which can be easily separated and interpreted in agarose gel electrophoresis. The primer described in this study can be used in further studies related to allele frequency researches and breeding strategies.
***Keywords**: Milk, κ-casein, CSN3, Polymorphism, PCR-RFLP, Primer design.*

Sığır Kappa-Kazeinin A ve B Genetik Varyantlarının PCR-RFLP Analizi için Yeni Bir Primer Dizaynı

Özet: İnek sütü yaklaşık %3,4 oranında yüksek kaliteli protein içerir ve insan beslenmesi için oldukça elzemdir. Kappa (κ) kazein, süt proteinlerinden birisidir ve *CSN3* geni tarafından kodlanmaktadır. Çalışmalar κ-kazeinin sütün özellikleri ve işlenmesi üzerine önemli bir etkiye sahip olduğunu göstermektedir. κ-kazein sütün kesilmesi sürecinde stabilize edici bir faktör olarak rol almaktadır; bu durum, κ-kazeini peynir fabrikalarında istenilir hale getirmektedir. Kappa (κ) kazeinin A ve B genetik varyantları iyi bilinmekte ve yoğun çalışılmaktadır. Sığırların genotiplenmesi için PCR-RFLP yöntemi kullanılmaktadır. Bu amaçla, farklı primer çiftleri *CSN3* geninin polimorfik bölgesini çoğaltmak için kullanılmaktadır. Önceki bir çalışmada, polimorfik bölgenin *HinfI* enzimi ile kesimi kısa ve benzer uzunlukta fragmentler oluşturdu. Agaroz jel elektroforezinde çok benzer DNA fragmentlerinin ayrımı neredeyse imkansızdır ve kısa DNA fragmentlerin yorumlanması bazen zorluklara sebep olmaktadır. Bu sebeple, bu çalışmada yeni bir primerin dizaynı tanımlandı. Yeni primerin kullanılamasıyla daha uzun DNA fragmenti başarılı bir şekilde çoğaltıldı ve bu PCR ürünlerinin *HinfI* kesimi, agaroz jel elektroforezinde kolaylıkla ayrılabilen ve yorumlanabilen çok farklı uzunlukta DNA fragmentlerinin oluşmasını sağladı. Bu çalışmada tanımlanan primer, allel frenkans araştırmaları ve yetiştirme stratejileri ile ilgili gelecek çalışmalarda kullanılabilir.
***Anahtar Kelimeler:** Süt, κ-kazein, CSN3, Polimorfizm, PCR-RFLP, Primer dizayn.*

Introduction

Bovine milk contains approximately 87% water and 13% dry matter including 4.6% lactose, 3.4% protein, 4.2% fat, 0.8% minerals and 0.1% vitamins and its composition can be affected by different factors such as breeding, feeding strategies, lactation stage and season (Lindmark-Månsson et al., 2003; Månsson, 2008). Approximately 80% of the milk proteins is consisted of casein proteins and the remaining part (~20%) is composed of whey proteins or serum proteins (β-lactoglobulins and of α-lactalbumin) (Eigel et al., 1984; Otaviano et al., 2005). Casein proteins are subdivided into four groups: αS1-casein, αS2-casein, β-casein, and κ-casein (Eigel et al., 1984). κ-casein protein comprises about 12% of the casein proteins. κ-casein gene (*CSN3*) has five exons and most of mature κ-casein protein is encoded by the fourth exon which indicates the importance of the fourth exon on κ-casein polymorphisms (Azevedo et al., 2008).

It has been reported that κ-casein is highly homologous to the γ chain of fibrinogen, which serves a similar function of the fibrinogen (Azevedo et al., 2008). Therefore, κ-casein plays a crucial role in the coagulation and curdling of milk in where it serves as a stabilizing factor (Fiat and Jolles, 1989). Until now, twelve genetic variants (*A, A', B, C, D, E, F^1, F^2, G^1, H, I* and *J*) of κ-casein have been identified in *Bos taurus* (Caroli et al., 2009), but the most frequent alleles are the A and B alleles (Farrell et al., 2004; Prinzenberg et al., 1999). In the variant A, threonine takes place at 136 of position on the

amino acid chain of κ-casein, and aspartic acid exists at 148 position on the chain. In the variant B, threonine is replaced with isoleucine and aspartic acid is replaced by alanine at the described positions of κ-casein (Alexander et al., 1988).

Studies have shown that milk yield and its composition, cheese yield and its quality are associated with κ-casein. Therefore, κ-casein is determined as a genetic marker and quantitative trait loci (Caroli et al., 2009; Comin et al., 2008). Furthermore Also, previous studies have shown that genetic variants of κ-casein have an impact on the manufacturing of milk and milk protein yield, protein content and, milk yield (McLean et al., 1984; Rachagani and Gupta, 2008; Tsiaras et al., 2005). B variant of κ-casein has been associated with shorter coagulation time and better curdles in cheese making (Aleandri et al., 1990; Marziali and Ng-Kwai-Hang, 1986). Marziali and Ng-Kwai-Hang (1986) have reported that the cheese yield from cows with genotype BB is 10% higher compared to cows with genotype AA. All these findings have led to κ-casein and its variant as a candidate for breeding strategies. For identification of A and B variant of *CSN3*, PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) approaches have been used. Patel et al. (2007) described a PCR-RFLP method for A and B variant of κ-casein. Patel et al. (2007) amplified 350 bp region of κ-casein by PCR, in which *Hinf*I restriction enzyme digestion of the PCR product resulted in short and very close length of DNA fragments: 84, 134 and 132 bp (Patel et al., 2007). Separation of 134 and 132 bp fragments in agarose gel electrophoresis is almost not possible and short fragments sometimes problematic. Therefore, the aim of the study was to improve the method by designing a more advantageous primer. Using the new primer, longer DNA region of κ-casein was amplified and restriction digestion of the amplified region resulted in more different DNA fragments, which are easily separated and interpreted in the agarose gel electrophoresis system.

Materials and Methods

DNA extraction: This study was approved by the Animal Researchers Local Ethics Committee of Van Yüzüncü Yıl University, Turkey (approval 07.03.2019-2019/2). Blood samples (2 ml) from fifteen cattle (Brown swiss) were collected in an anti-coagulant tube containing EDTA and stored at -20˚C until DNA isolation. DNA isolation was carried out using the WizPrep™ gDNA Mini Kit (Wizbio, Republic of Korea) following the manufacturer's protocol with some modification. Briefly, frozen blood samples were thawed in room temperature and 200 µl whole blood was added to the microcentrifuge tube. Then, the tubes were centrifuged at 10.000 *g* for 1 min and supernatants were discarded, and pellets were washed twice by nuclease-free water. The following steps were performed as described in the protocol. DNA concentrations were measured spectrophotometrically.

Primer design: DNA sequence of *CSN3* was downloaded from the GenBank (No: AY380228) and focused on the fourth exon of *CSN3* (Figure 1). To obtain easily separable restriction fragments in agarose gel electrophoresis, 25 bp sequence was randomly selected, which is 100 bp upstream region of the previously described forward primer by Patel et al. (2007).

Figure 1. DNA region of the interest of *CSN3*. Gray highlight shows the region of the fourth exon of *CSN3*. Yellow highlights show binding sites of the previously described primers by Patel et al. (2007). Green highlight shows binding site of the new primer.

The selected sequence was analyzed by Primer-Blast (Ye et al., 2012) and the OligoAnalyzer (Owczarzy et al., 2008) tools for primer specify and property to determine whether the primer is useful for PCR amplification or not. For the calculations in OligoAnalyzer tool, default values were considered. To check any existing cutting site on the interested 100 bp upstream DNA region, the online restriction digest tool RestrictionMapper (version 3) (http://www.restricti°nmapper.org/) was used.

PCR: The polymorphic region of the *CSN3* gene was amplified by using forward (called Primer 1) 5'-ATCATTTATGGCCATTCCACCAAAG-3' and reverse 5'-GCCCATTTCGCCTTCTCTGTAACAGA-3' (called Primer 2) primer pair, which amplifies 350 bp region described by Patel et al. (2007). Also, the polymorphic region was amplified by the newly designed forward primer (called Primer 3) 5'-CTGCCCAAATTCTTCAATGGCAAGT-3' and Primer 2 to amplify 450 bp region of DNA. PCR amplification was carried out using 2xTaq Master Mix (Vivantis, Malaysia) in the presence of ~100 ng template DNA and 0.2 µM (final concentration) of each primer. PCR condition for Primer 1 and Primer 2 was included: an initial denaturation step at 94°C for 2 min. and 30 sec., followed by 35 cycles of 94°C for 20 sec. for denaturation, 55°C for 1 min. for

annealing, 72°C for 40 sec. for extension, and a final extension at 72°C for 5 min. The second PCR condition for Primer 3 and Primer 2 was the same with the first condition except for annealing condition which was 60°C for 40 sec. PCR products were run in 2% agarose gel containing fluorescent dye (Eur°Clone, Italy) at 90 volts (V) for 70 min. After electrophoresis, the gel was visualized under ultraviolet (UV) light and recorded.

Restriction of PCR product: The amplified (both 350 and 450 bp) PCR products (about 20 µl) were digested overnight by the *Hinf*I restriction enzyme (Fermentas) in a water bath at 37°C. After the overnight digestion, digested products were separated in 2% agarose gel containing fluorescent dye (Euroclone, Italy) at 90 V for 70 min and visualized under UV light.

Results

Primer specificity of the newly designed primer (Primer 3) was checked by the Primer-Blast tool (Ye et al., 2012) in this study. Results showed that Primer 3 and the previously described reverse primer (Primer 2) by Patel et al. (2007) was able to amplify the specific 450 bp region of *CSN3,* which comprises single nucleotide polymorphisms (SNPs), causing A and B variant proteins (data not shown).

The online tool RestrictionMapper (version 3) was used to show any existing restriction site of *Hinf*I on the 100 bp upstream region. 450 bp DNA sequence was uploaded and then cutting site and probable restriction fragment was obtained for *Hinf*I *in silico* (Figure 2). As shown in Figure 2, there was no existing restriction site for *Hinf*I, and expected DNA fragments were 85, 131, and 234 bp, which can be easily separated in conventional agarose gel electrophoresis.

Melting temperature (Tm) and Guanin-Sitozin ratio (GC) of primers were shown in Table 1. Primer 3, has a better Tm and GC content compared to the described forward primer by Patel et al. (2007), which indicates usefulness of the Primer 3 in PCR.

450 bp digest

Enzymes: HinfI

Length	5' Enzyme	5' Base	3' Enzyme	3' Base	Sequence
234	none	1	HinfI	234	CTGCCCAAAT TCTTCAATGG CAAGTTTTGT CAAATACTGT GCCTGCCAAG TCCTGCCAAG CCCAGCCAAC TACCATGGCA CGTCACCCAC ACCCACATTT ATCATTTATG GCCATTCCAC CAAAGAAAAA TCAGGATAAA ACAGAAATCC CTACCATCAA TACCATTGCT AGTGGTGAGC CTACAAGTAC ACCTACCACC GAAGCAGTAG AGAGCACTGT AGCTACTCTA GAAG
131	HinfI	235	HinfI	365	ATTCTCCAGA AGTTATTGAG AGCCCACCTG AGATCAACAC AGTCCAAGTT ACTTCAACTG CAGTCTAAAA ACTCTAAGGA GACATCAAAG AAGACAACGC AGGTAAATAA GCAAAATGAA TAACAGCCAA G
85	HinfI	366	none	450	ATTCATGGAC TTATTAATAA AATCGTAACA TCTAAACTAG CGTAGATGGA TAAATTAAAT CTGTTACAGA GAAGGCGAAA TGGGC

Figure 2. *Hinf*I restriction mapping of predicted 450 bp PCR product. The sequence corresponds to A variant of *CSN3* gene (GenBank No: AY380228).

Table 1. In this study used primers and its properties.

Primer	Sequence (5'>3')	Length	Tm (°C)*	GC (%)	Reference
Primer 1	ATCATTTATGGCCATTCCACCAAAG	25	56 .0	40.0	Patel et al. (2007)
Primer 2	GCCCATTTCGCCTTCTCTGTAACAGA	26	60.7	50.0	Patel et al. (2007)
Primer 3	CTGCCCAAATTCTTCAATGGCAAGT	25	58.5	44.0	In this study

*Tm values were calculated based on default setting on OligoAnalyser tool.

For secondary structure formation properties of primers, the most probable hairpin structures and its ΔG (Gibbs free energy) and Tm value was determined (Figure 3). According to the results, there was no significant difference between Primer 1 and Primer 3 in the case of ΔG and Tm values.

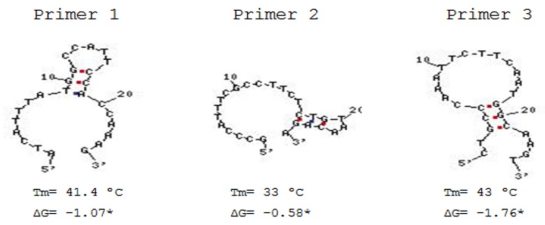

Figure 3. Predicted hairpin structures of primers. The most possible structures were shown. Calculations were performed by default setting. * indicates kcal.mole^{-1}

Figure 4. Self-dimer formation properties of primers. Solid line represents pairs of complementary bases and dotted lines represent additional complementary bases, which does not impact on calculated ΔG values. * indicates kcal.mole^{-1}.

Self-dimer structure formation analysis indicates the binding of primer each self. The most probable self-dimer structures were determined for each primer (Figure 4). As shown in the figure**,** Primer 1 has eight complementary pairs and its ΔG value is significantly lower negative than that of Primer 3, which supports usefulness of Primer 3 and also indicates less problematic PCR experience, In the next step, hetero-dimer formation was checked and the most probable hetero-dimer formation was determined (Figure 5). Results indicated that Primer 3 and Primer 2 pairs have a sight negative ΔG value than that of Primer 1 and Primer 2.

Figure 5. Hetero-dimer properties of primer pairs. Solid line represents pairs of complementary bases, and dotted lines are additional complementary bases and do not impact on calculated ΔG values. * indicates kcal.mole^{-1}.

After primer specificity and properties were checked, PCR amplifications of the interested region were carried out using the described primers. PCR products were run in 2% agarose gel and then PCR products were digested overnight by *Hinf*I restriction enzyme. Restriction fragments were separated by agarose gel electrophoresis as described (Figure 6). As expected, Primer 2 and Primer 3 were able to amplify 450 bp region of *CSN3,* and *Hinf*I restriction of the 450 bp PCR product resulted in 85, 131 and 234 bp fragments for genotype A, 365 and 85 bp for genotype B, 85, 131, 234 and 365 bp for genotype AB (Figure 6). It is clearly shown that the enzyme digested fragments were easily separated in 2% agarose gel electrophoresis.

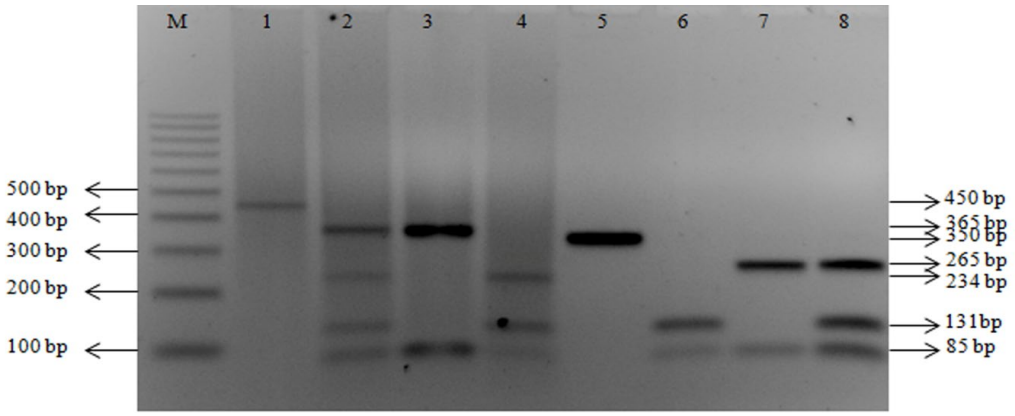

Figure 6. Agarose gel electrophoresis of PCR product and its restricted fragments by *Hinf*I. Line M indicates 100 bp DNA marker (MR61, Blirt, Poland). Line 1 shows PCR product, which obtained using Primer 2 and Primer 3. Line 2, line 3 and line 4 shows genotype AB, B and A, respectively, which were obtained by *Hinf*I digestion of the 450 bp PCR product. Line 5 indicates 350 bp PCR product, which is obtained described primer by Patel et al. (2007). Line 6, line 7 and line 8 show genotype A, B and AB, respectively, which are obtained by *Hinf*I digestion of the 350 bp PCR product.

Discussion

In literature, to determine DNA variations, there are many different methods such as sequencing, denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP) and restricted fragment length polymorphism (RFLP) (Dong and Zhu, 2005; Fodde and Losekoot, 1994; Franca et al., 2002; Ota et al., 2007). Sequencing gives more detailed information

about of interest compared to other methods, but it is more costly and requires automated equipment. In the DGGE method, denaturing gradient gel is needed to be prepared, which are required especial material and equipment. In the SSCP method, the polyacrylamide gel electrophoresis technique is used, which is more complex compared to the agarose gel electrophoresis technique. Also, both DGGE and SSCP methods do not show a mutation type. They can only show existing mutation(s). However, the PCR-RFLP method is simpler than the other methods based on the required equipment and applications, and it may give information regarding mutation type. Thus, it has a big advantage for genotyping studies and has been used for many decades. In this study, a restriction enzyme-based detection of single nucleotide polymorphisms of *CSN3* was described, designing more advantageous primer for PCR-RFLP analysis.

PCR is used for synthesizing of a million copies of specific DNA fragments and it is one of the most widely used tools in health and life science researches (Saiki et al., 1988). Secondary structures such as self-dimers, hairpins, and heterodimers of the designed primer or primers may affect the success of PCR. Therefore, these parameters might be considered to design a primer. *ΔG* is the Gibbs free energy for oligonucleotide as calculated by the nearest neighbor method (Breslauer et al., 1986; Sugimoto et al., 1996). *ΔG* indicates energy to disrupt the duplex formed oligonucleotide and determines the stability of the DNA molecule (Breslauer et al., 1986; Stephenson, 2003). It is considered that *ΔG* value of primer(s) should be weaker or higher value than -9.0 kcal/mole for acceptable secondary structure(s). *ΔG* value with higher value than -9.0 kcal/mole indicates the quality of primer, in where quality is the ability to efficiently amplify the desired template (Mann et al., 2009). Primer 3 has acceptable *ΔG* values, which indicates that it is an appropriate candidate for amplification of the region of interest (Figure 4 and Figure 5).

DNA melting is the process in which a double-stranded helix DNA is separated into two single strands DNA by denaturants (Rouzina and Bloomfield, 1999). Tm of double-stranded DNA or oligonucleotide is that half of the molecule is double-stranded conformation and half is single-stranded form in the defined temperature (Stephenson, 2003). Melting temperatures of two primers should be similar for an optimal PCR reaction (Stephenson, 2003). It is considered that Tm difference between primers should be lower than 5. As shown in Table 1. Tm value of Primer 3 and Primer 2 was very close to each other, which indicates usefulness of the primer pair. Tm value is affected by presence of potassium (K+) and magnesium (Mg++) ions. Therefore, for better calculation of Tm, the salt presence should be considered (Stephenson, 2003).

*Hinf*I was isolated from the *Haemophilus influenza* type I. The restriction site of *Hinf*I is 'G/ANTC' in where N might be a A, C, G or T bases (Smith and Wilcox, 1970). As shown in Figure 2 and 6, Allele A has two *Hinf*I restriction site, which is 'G/ATTC'at 234 and 365 bp on the region of interest and its restriction results in 85, 131, and 234 bp fragments. However, the 350 bp amplicon obtained by described primers by Patel et al. (2007) results in 84, 132, and 134 for A variant. As shown in Figure 6, only two fragments were shown due to the close length fragment issue. In this study, using the new forward primer let to a more practical evaluation of the genotypes since longer and more different fragments are advantageous due to better band intensity, requiring lower percentage agarose or/and running time.

In conclusion, the described primer in this study results in easily separable and longer restriction fragments, which leads to a more easy and practical evaluation of the genotypes in conventional agarose gel electrophoresis. It can be easily used for allele frequency research of *CSN3* and also for breeding strategies considering A and B variant of *CSN3*.

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