Detection of B κ-casein variant (CSN3*B) in Burlina dairy cattle by PCR-TTGE

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Dairy Sci. Technol. 88 (2008) 217–223

DOI: 10.1051/dst:2007018

Available online at:
www.dairy-journal.org

Original article

Detection of B κ-casein variant (*CSN3*B*) in Burlina dairy cattle by PCR-TTGE

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Received 6 June 2007 – Accepted 10 September 2007

Abstract – The aim of this study was to develop and optimize a PCR-TTGE method for the detection of B κ-casein variant (*CSN3*B*), which is known to play a major role in cheese technology. The effects of the different κ-casein alleles on the quality and the quantity of cow's milk have been widely reported and the availability of accurate and reliable protocols for the identification of the most common alleles is of great interest in breeding projects. In the present study a new genomic DNA extraction method from milk samples and a PCR-TTGE protocol to quickly identify A and B κ-casein allelic variants were developed. The DNA extraction method was fast and suitable for DNA amplification, while TTGE proved to be a powerful technique for discrimination of A and B alleles. This method was applied to 197 milk samples derived from Burlina, an Italian dairy cattle breed. *CSN3*B* allele frequency as detected by PCR-TTGE was 0.368. The development of this PCR-TTGE protocol could be used for future breeding programs to improve milk coagulation ability traits.

κ-casein / milk / PCR-TTGE / dairy cattle / Burlina breed

摘要 – 利用 PCR-TTGE 方法检测 Burlina 奶牛中 κ-酪蛋白 B 变异体 (*CSN3*B*)。本研究目的是建立和优化一种检测 κ-酪蛋白 B 变异体 (*CSN3*B*) 的 PCR-TTGE 方法。κ-酪蛋白 B 变异体 (*CSN3*B*) 在干酪生产中具有重要的意义。κ-酪蛋白不同等位基因对牛奶质量与产量的影响已被广泛报道。建立用于鉴定 κ-酪蛋白共有等位基因的正确可靠方法, 已经成为饲养业关注的研究热点。本研究建立了一种从牛奶样品中提取基因组 DNA 的新方法以及利用 PCR-TTGE 技术快速鉴定 κ-酪蛋白A、B等位基因变异体的方法。该 DNA 提取方法具有快速且适用于 DNA 的扩增, 同时 TTGE 是区分A、B等位基因变异体的有效技术手段。应用上述方法对来自印度 Burlina 种奶牛的 197 份牛奶样品进行了检测。利用 PCR-TTGE 检测出 *CSN3*B* 等位基因的频率为 0.368。本研究建立的 PCR-TTGE 方法适用于对未来通过饲养方法来改善牛奶凝结特性的研究。

κ-酪蛋白 / 牛奶 / PCR-TTGE / 奶牛 / Burlina种

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Résumé – Détection par PCR-TTGE d'un variant B de caséine κ (*CSN3*B*) dans la race de vaches laitières Burlina. Le but de cette étude était de développer et optimiser une méthode PCR-TTGE pour la détection du variant B de la caséine κ (*CSN3*B*) connu pour son rôle majeur en technologie fromagère. Les effets des différents allèles de caséine κ sur la qualité et la quantité de lait ont été largement décrits et la disponibilité de protocoles précis et fiables pour l'identification des allèles les plus communs présente un grand intérêt pour les projets de sélection. Dans la présente étude, une nouvelle méthode d'extraction d'ADN génomique à partir d'échantillons de lait et un protocole PCR-TTGE pour identifier rapidement les variants alléliques de caséine κ A et B ont été développés. La méthode d'extraction d'ADN s'est avérée rapide et convenable pour l'amplification d'ADN tandis que la TTGE permettait de discriminer efficacement les allèles A et B. Cette méthode a été appliquée à 197 échantillons de lait de vaches de race italienne Burlina. La fréquence de l'allèle *CSN3*B* détecté par PCR-TTGE était de 0,368. Le développement de ce protocole PCR-TTGE pourrait être utilisé dans de futurs programmes de sélection pour améliorer le caractère d'aptitude du lait à la coagulation.

caséine / lait / PCR-TTGE / vache laitière / race Burlina

1. INTRODUCTION

Several genetic variants have been described for bovine κ-casein [5, 9], of which the most common are A and B variants differing by the substitution of two amino acids at positions 136 and 148 [8]. A positive association between the B variant of κ-casein and the technological properties of milk has been reported. In milk a high content of B variant compared with A is associated with higher casein content and a better cheese-yielding capacity [10, 13]. Therefore, the availability of accurate and reliable protocols for the identification of κ-casein genotypes still remains of great interest in industrial and breeding projects aimed at improving the cheese-making properties of milk. Many techniques have so far been developed in order to distinguish the genetic variants of caseins, especially those of importance in cheese technology such as β-casein and κ-casein [19]. Typing of milk proteins can be performed by electrophoretic separation of milk proteins directly from milk samples. SSCP [1, 9], PCR-RFLP analysis [7, 15] and, very recently, microarrays [4, 6] are DNA-based techniques which have been suggested for κ-casein allele discrimination. DNA extraction can be performed from different types of samples (milk, blood, semen) and genotyping could be applied to cows independently of sex, age or physiological lactation stage. TTGE (Temporal Temperature Gradient Gel Electrophoresis) is an interesting method based on electrophoretic separation of DNA molecules that have the same length but a different nucleotide sequence. In this technique, PCR-amplified double-stranded DNA is subjected to electrophoresis under the denaturating conditions obtained by a temperature gradient; migration depends on the degree of DNA denaturation [3].

The aim of this work was to develop and optimize a PCR-TTGE method for the detection of the B κ-casein variant in the Burlina population, an indigenous Italian cattle breed well adapted to the Alpine mountain area of the North East of Italy.

2. MATERIALS AND METHODS

2.1. DNA extraction from milk

In order to study the genetic polymorphism at the κ-casein locus in Burlina cattle, milk samples were collected from 197 Burlina cows, which, in 2005, represented the total number of Burlina cows in the lactation stage in the Veneto Region, Italy. The samples were kept maintained refrigerated at 4 °C and processed within 2 days after collection.

DNA was extracted from individual milk samples according to a method developed in the present study. Fifteen-mL milk samples were gently mixed with an equal volume of isolation buffer (200 mmol·L^{-1} EDTA, 20 mmol·L^{-1} Tris-HCl, 0.5 mol·L^{-1} NaCl, pH 7.4), maintained at room temperature for 10 min and centrifuged at 12 °C for 5 min at 3000× *g*. The pellet was subjected to a washing step in $T_{10}E_{100}$ buffer (100 mmol·L^{-1} EDTA, 10 mmol·L^{-1} Tris-HCl, pH 7.4) and then resuspended in 550 μL of $T_{50}E_{20}$ buffer (20 mmol·L^{-1} EDTA, 50 mmol·L^{-1} Tris-HCl, pH 7.4). The 550-μL aliquots were transferred into a new 2-mL tube, supplemented with 200 μL of SDS 10% (w/v) and incubated at 65 °C for 30 min at 700 rpm in a thermomixer (Thermomixer Comfort, Eppendorf, Hamburg, Germany). After addition of 275 μL of potassium acetate 5 mol·L^{-1} and incubation on ice for 10 min, the DNA was recovered by an isopropanol/ethanol precipitation [12]. The DNA was finally resuspended in TE (10 mmol·L^{-1} Tris-HCl, 1 mmol·L^{-1} EDTA, pH 7.4) at 60 °C to facilitate the nucleic acid solubilization.

2.2. Primers and PCR

The accession number of the genetic sequences used for the preliminary in silico study were GenBank AY380228 and AY380229 for the *CSN3*A* and *CSN3*B* alleles, respectively. ClustalW software [17] was used for sequence aligment.

To amplify a 165-bp region of the κ-casein exon IV, the primer pair Csn-F (GCTAGTGGTGAGCCTACAAGTACA) and Csn-R (GTTGTCTTCTTTGATGTCTCCTTAGAG) were developed. As required by TTGE analysis [14], a third primer, Csn-R-GC (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGTTGTCTTCTTTGATGTCTCCTTAGAG) containing a GC-clamp was generated by adding a 5' GC tail of 39 bases to Csn-R. The Bio-Rad WinMelt 2.0.13 software was used to study the melting profile according to the manufacturer's instructions.

Amplification reactions were carried out in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany) in a 50-μL volume containing 0.2 mmol·L^{-1} of dNTPs, 1 X of reaction buffer (10 mmol·L^{-1} Tris-HCl, 1.5 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} KCl and 0.1% Triton X-100), 1.5 U of DyNAzyme Taq polymerase (Celbio, Milano, Italy), and 0.2 μmol·L^{-1} of each primer. The amplification conditions for the Csn-F/Csn-R-GC primers used in TTGE experiments consisted of 35 cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 60 °C, and extension for 1 min at 72 °C. The first cycle was preceded by an initial denaturation step of 4 min at 94 °C, and the last cycle was followed by a final extension step of 5 min at 72 °C. In the case of milk samples with low amplification signals, PCR sensitivity was enhanced using a nested PCR-protocol based on a first amplification with the primers pair and conditions (except the elongation time reduced to 1 min) previously described by Barroso et al. [1], followed by a second amplification with the Csn-F/Csn-R-GC primers developed in the present study.

2.3. TTGE

The DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) was used for TTGE analysis. Polyacrylamide gels consisted of 8% (v/v) polyacrylamide (37.5:1 acrylamide/bis-acrylamide), 7 mol·L^{-1} urea and 1.25 X Tris-acetate buffer [12]. Before loading, the wells were washed twice with the running buffer (1.25 X Tris-acetate). The thermal protocol used in TTGE separation was: 10 min at 55.8 °C

at 60 V followed by a 3-h ramp from 55.8 °C to 57 °C at 180 V with a rate of 0.4 °C·h^{-1}. After the electrophoresis, gels were stained in a solution containing 0.5 μg·mL^{-1} ethidium bromide. To verify the identity of the TTGE bands, blocks of polyacrylamide gels containing selected bands were recovered with a sterile cutter. The pieces were transferred into 100 μL of sterile distilled water and the DNA of the bands was left to diffuse overnight at room temperature. Two μL of the eluted DNA were used for re-amplification with the primers Csn-F/Csn-R-GC and then submitted to sequencing at BMR Genomics (Padova, Italy).

3. RESULTS AND DISCUSSION

In the present study a new DNA isolation method which allows the extraction of eukaryote DNA from raw milk, without using toxic reagents or time-consuming and expensive enzymatic reactions to eliminate the protein fraction, was developed. The new extraction method takes advantage of the better solubility of α and β-caseins in low bivalent ion solutions, obtained by adding a Ca^{2+} chelating agent, than in natural milk suspensions. Thus, by using a high concentration of EDTA buffer it was possible to eliminate most of the proteins and more easily collect somatic cells. The increased solubility of α and β-caseins in low concentrations of bivalent ions is presumably related to the subtraction of Ca^{2+} ions, which causes the modification of micellar equilibrium [11, 18].

The GC composition of the Csn-F/Csn-R 165-bp fragments derived from the amplification of A and B alleles was 44.85% and 45.45% respectively, while the percentage of DNA sequence identity was 97.58%. The Csn-F/Csn-R amplification product of allele B differs from the A product by four nucleotide polymorphisms in the positions 32 (Ile_{136} instead of Thr_{136}), 68 (Ala_{148} instead of Asp_{148}), 129 (silent mutation) and 137 (non-coding region) of their amplified fragments (Fig. 1). In the PCR-TTGE protocol developed in the present study, two bands, which were identified by DNA sequencing as A and B alleles, could be distinguished; furthermore, a third TTGE band identified as E allele was detected in 11 out of the 197 milk samples (Fig. 2). The GC composition of the amplified E allele fragment (GenBank AF041482) was 45.45%, while the percentage of DNA sequence identity with alleles A and B was 99.39% and 96.67%, respectively. In particular, one single polymorphism was present at position 88 (Gly_{155} instead of Ser_{155}) of the E variant amplified fragment in comparison with A (Fig. 1). The different migration patterns of A, B and E amplicons cannot be only explained by the different GC percentage of their sequences (A in comparison with B and E) but also by the presence of specific nucleotide substitutions which do not change the GC percentage but modify the migration pattern (B in comparison with E). It is interesting to note that the TTGE conditions adopted in this study are very powerful and are able to discriminate even fragments such as A and E, which differ by only one base exchange (one transition from A to G at position 88).

The optimized voltage values and range of temperatures of the developed TTGE protocol allowed the discrimination of A, B and E alleles in only 3 h of electrophoretic running. Furthermore, since TTGE allows the simultaneous evaluation of many samples (up to 40), it could be a useful and time-effective method to be applied in population studies.

Genotype frequencies revealed by PCR-TTGE analysis in Burlina dairy cattle were 35.0%, 47.7%, 11.7%, 3.1% and 2.5% for AA, AB, BB, AE and BE, respectively, while no EE genotype was detected (Tab. I). The frequencies of alleles A and B were 0.604 and 0.368, while a frequency

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                    Ala126                                  Thr136
CSN3*A, AY380228    GCTAGTGGTG AGCCTACAAG TACACCTACC ACCGAAGCAG TAGAGAGCAC

                                                            Ile136
CSN3*B, AY380229    .......... .......... .......... .T........ ..........

CSN3*E, AF041482    .......... .......... .......... .......... ..........

                                      Asp148                          Ser155
CSN3*A, AY380228    TGTAGCTACT CTAGAAGATT CTCCAGAAGT TATTGAGAGC CCACCTGAGA

                                      Ala148
CSN3*B, AY380229    .......... .......C.. .......... .......... ..........

                                                                  Gly155
CSN3*E, AF041482    .......... .......... .......... .......G.. ..........

                                                  Val169
CSN3*A, AY380228    TCAACACAGT CCAAGTTACT TCAACTGCAG TCTAAAAACT CTAAGGAGAC

CSN3*B, AY380229    .......... .......... ........G. ......T... ..........

CSN3*E, AF041482    .......... .......... .......... .......... ..........

CSN3*A, AY380228    ATCAAAGAAG ACAAC

CSN3*B, AY380229    .......... .....

CSN3*E, AF041482    .......... .....
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Figure 1. Alignment of the Csn-F/Csn-R 165-bp amplification fragments derived from *CSN*A*, *CSN*B* and *CSN*E* κ-casein variants. Alleles and GenBank accession numbers are shown; each identical nucleotide in the sequence is reported as a dot. The amplified fragment corresponds to the κ-casein A protein region Ala_{126} to Val_{169}; aminoacid variations in κ-casein B and E are reported.

Table I. κ-Casein genotypes and allelic frequencies determined by PCR-TTGE.

Detected genotypes							
	Cows	A/A	B/B	E/E	A/B	A/E	B/E
n	197	69	23	0	94	6	5
%	100	35.0	11.7	0.0	47.7	3.1	2.5
Detected allelic frequencies							
	A			B		E	
	0.604			0.368		0.028	

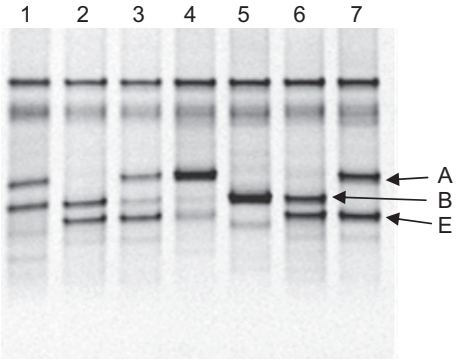

Figure 2. Negative image of TTGE analysis of *CSN3* amplification products. Genotypes: lane 1 A/B; lanes 2 and 6 B/E; lanes 3 and 7 A/E; lane 4 A/A; lane 5 B/B.

of 0.028 was found for allele E. Deviation between observed genotypic frequencies and those expected under Hardy-Weinberg equilibrium were not significant ($p > 0.05$; data not shown), suggesting that the Burlina population considered is in equilibrium for the *CSN3* locus and no selective advantage for any of the genotypes is present.

The predominance of the *CSN3*A* variant in Burlina is comparable with previous results reported for Italian Fresian [16] and Italian Holstein [2] cattle.

4. CONCLUSION

The PCR-TTGE protocol developed in the present work may be a useful and reliable tool to evaluate polymorphism at the bovine κ-casein locus and to easily detect B κ-casein genotypes in industrial and breeding plans aimed at improving the cheese-making properties of milk. By modifying the DNA extraction protocols, the system, which was applied to the study of genetic variants of Burlina dairy cattle starting from milk samples, could be extended to other types of samples, such as blood and semen, and recommended for genotyping of larger populations, including cows which are not in the lactation stage or bulls.

Acknowledgements: This work was financially supported by the project of the Veneto Region, Italy, "Recupero della popolazione autoctona Burlina per la salvaguardia delle risorse genetiche e la qualificazione dei prodotti lattiero caseari". We thank Dr. C. Ossensi for collecting milk samples.

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