Method Article

A novel test system for genotyping rs43703016 single-nucleotide substitutions in the bovine *CSN3* gene

ABSTRACT

Aims: Caseins are among the main milk proteins that determine many of its properties. Bovine kappa-casein (CSN3) is associated with the qualitative composition of milk, as well as with the quality of cheese obtained from this milk. The rs43703016 single-nucleotide substitution (g.88532332A>C; Asp148Ala) in exon 4 of the bovine *CSN3* gene plays an important role in the production of quality hard cheeses. Various methods for the DNA testing of this substitution have been developed in the last three decades. Emergent DNA technologies provide an opportunity to modernize methods of genotyping single-nucleotide polymorphisms.

Results: We have developed and verified a method to differentiate A/C alleles of the rs43703016 substitution in the bovine *CSN3* gene by real-time PCR using allele-specific fluorescent probes.

Conclusion: Our new method allows fast genotyping of animals, and may be used for selection of cows carrying the CC genotype, which determines good cheese-making properties of milk.

Keywords: cattle, CSN3 gene, rs43703016 single-nucleotide, genotyping, real-time PCR

1. INTRODUCTION

Marker-assisted selection in animal breeding is based on genotype knowledge and selection of animals with genetic data that are best from the industrial point of view. In this respect, particular attention has been paid to milk proteins, in particular caseins [1]. The *CSN3* gene encoding kappa-casein has been receiving the most attention. Several substitutions in this gene have been identified and described [2]; and 3 of them affect the characteristics of milk: rs43703015 [3], rs43703016 [4, 5] and rs43703017 [3]. From a practical point of view, the rs43703016 substitution (g.88532332A>C; Asp148Ala) is of particular interest. It should be mentioned that the alleles of this polymorphism were denoted in the literature with the letters A and B (nucleotides A and C, respectively) until recently. The kappa-casein complex haplotypes, 136Thr(A)/Ile(B) and 148Asp(A)/Ala(B), are also called alleles A and B [6].

This substitution stirred an interest long ago, when the alleles were shown to affect the content of milk proteins and the quality of cheese [7–10], and this interest is still displayed [3, 11–16]. The C allele of this polymorphism is industrially valuable.

The first DNA test systems for allele testing of this substitution first appeared approximately 30 years ago [17]. Novel test systems emerged with the modernization of PCR technology and development of new methodological approaches; they use amplification of fragments of several genes and hybridization with variant-specific probes [patent WO2007107862A2, 2007], tetra-primer PCR [patent RU2386700C1, 2010], amplification and polymorphism

assessment based on the melting temperature of PCR products of the CSN3 and LGB genes [patent CZ303083B6, 2012], allele-specific PCR [patent RU2013106990A, 2014], and real-time PCR for allele testing of 3 substitutions in the gene at the same time [patent RU2646140C1, 2018]. The latter test system is the most effective one; however, it does not allow assessment of the cis-trans positions of the substitutions in question. As can be concluded, the interest in the development of methods that can be used for allele testing of the rs43703016 substitution in the *CSN3* gene is not waning. A simple, fast, and inexpensive method for mass genotyping for this polymorphism in cattle is needed.

The objective of the reported study was to develop and verify a method to differentiate A/C alleles of the rs43703016 substitution in the bovine *CSN3* gene by real-time PCR (RT-PCR).

2. MATERIAL AND METHODS

The study was conducted on 94 samples obtained from black-and-white Holsteinized cows of the Pravda-N LLC animal farm (Dzerzhinsk District, Kaluga Region).

2.1 DNA isolation and oligonucleotide design

DNA was isolated from 94 whole blood samples obtained from black-and-white Holsteinized cattle using an M-sorb kit (Sintol, Russia).

The nucleotide sequences of the primers and probes were selected with the help of the GeneRunner and Multiple primer analyzer software (https://www.thermofisher.com/). The primers and probes were synthesized by DNA-synthesis LLC (Moscow, Russia).

2.2 Real-time PCR

The amplification reaction was performed in 10 µL of a PCR mixture containing 5 µL of the LightCycler® 480 Probes Master reagent (Roche, Switzerland), a mixture of the straight primer k-casein-F: 5'-CCATCGAAGCAGTAGAGAGCA-3' and the reverse primer k-casein-R: 5'-GGACTGTGTTGATCTCAGGTGG-3' (10 µM), 0.4 µM of each of the allele-specific probes, k-casein-C: 5'-FAM-CTACTCTAGAAGCTTCTCCAG-BHQ1-3' and k-casein-A: 5'-VIC-CTACTCTAGAAGATTCTCCA-BHQ1-3', and 10 ng of the DNA. PCR was carried out using a LightCycler 96 device (Roche, Switzerland) under optimized conditions (initial denaturation at 95 °C for 10 min followed by 40 cycles of amplification: 95°C for 20 s, 55°C for 30 s, 72°C for 20 s). Fluorescence was detected at the elongation stage in the FAM and VIC channels. Results were analyzed with the LightCycler® 96 version SW1.1 amplifier software (Roche, Switzerland).

2.3 Method validation

Validation was performed with PCR-RFLP. The PCR utilized 0.2 µM portions of the primers k-cas-D: 5'-ATAGCCAAATATATCCCAATTCAG-3' and k-cas-R: 5'-TTTATTAATAAGTCCATGAATCTT-3', Fusion Hot Start II High-Fidelity DNA polymerase kit (Thermo Scientific, USA), used in accordance with the manufacturer's instructions and 30 ng of the genomic DNA. The PCR was performed using a T100 amplifier (Bio-Rad, USA) under the following conditions: pre-denaturation at 98°C for 30 s, 40 amplification cycles at - 98°C - 10 s, at 55°C - 30 s, at 72°C - 1 min. The restriction was performed in 20 µL of a mixture containing 2 µL of a 10x buffer and 1 unit of Hind III endonuclease (Thermo Scientific, USA); obtained amplicons were processed for 16 hours at 37°C. The restriction results were assessed using electrophoresis in 1.2% agarose gel.

The occurrence rates of identified genotypes were calculated by direct counting.

3. RESULTS AND DISCUSSION

In this study, we developed a new test system for genotyping rs43703016 single-nucleotide substitutions in exon 4 of the kappa-casein gene (*CSN3*) using real-time PCR with allele-specific fluorescent probes.

The developed test system utilized two primers common to both alleles of the *CSN3* gene and two allele-specific TaqMan probes labeled with FAM and VIC reporter dyes. The k-cas-

D and k-cas-R primers are used to amplify an 85 bp fragment of the *CSN3* gene. Identification of alleles C and A of the rs43703016 substitution in the *CSN3* gene is based on comparison of the fluorescence intensities of the FAM and VIC reporter dyes, respectively. The genotyping analysis was carried out using the LightCycler® 96 version SW1.1. amplifier software (Figure 1).

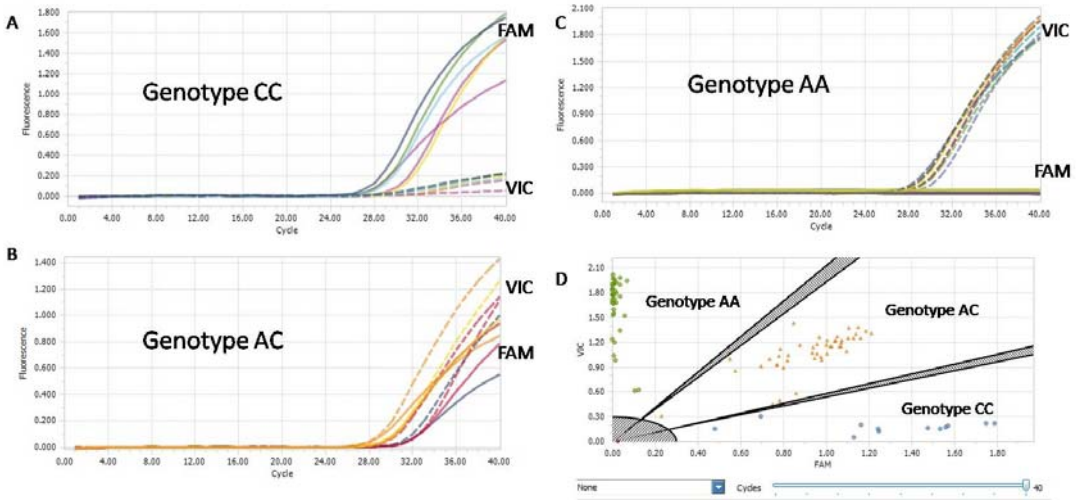

Fig. 1. Example of the detection of allele variants C and A of the rs43703016 substitution in the bovine CSN3 gene by real-time PCR. Fluorescence curves (A-C) and the genotype distribution (D) are presented.

CC cows were found to have an increase in fluorescence signal in the FAM channel (Figure 1A). In AA cattle, the fluorescence signal was registered in the VIC channel (Figure 1C). Heterozygous cows (genotype AC) had signal detected in both channels, FAM and VIC (Figure 1B). Therefore, RT-PCR results obtained with allele-specific TaqMan probes allow detection of the presence in the analyzed DNA sample of each of the studied alleles (C and A) of the rs43703016 substitution in the *CSN3* gene and, accordingly, determination of the animal's genotype.

The proposed real-time PCR-based test system was validated on 94 DNA samples obtained from black-and-white Holsteinized cattle. The genotyping results revealed that 49% of the animals were carriers of both alleles (genotype CA), 38 % of the cows were allele A homozygotes (genotype AA), and 13% of the cattle were allele C homozygotes (genotype CC). These results are generally consistent with available literature data on the frequencies of these genotypes in cattle populations (e.g., [6]) or information from ENSEMBL genome database: AC 37.5%; AA 50.0%; CC 12.5% [https://www.ensembl.org/].

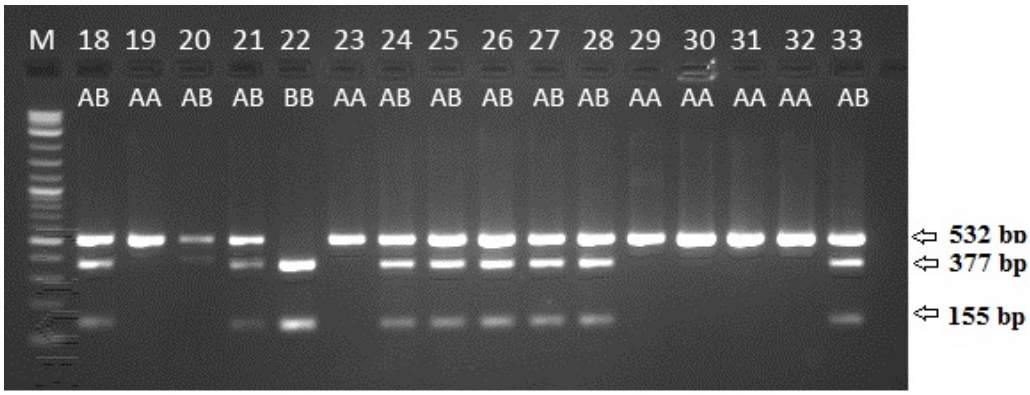

Fig. 2. An example of cattle genotyping results with regard to alleles A/C of the rs43703016 substitution in the *CSN3* gene.

The proposed method was validated using PCR-RFLP analysis [18]. Results obtained with the two genotyping methods completely coincided; however, the method proposed in this paper allows significant (up to 1 hour) reduction of the assay time, which gives it an advantage over PCR-RFLP analysis. Additionally, the use of RT-PCR allows to avoid an electrophoresis stage and, consequently, contamination of the laboratory with amplification products of the *CSN3* gene fragment.

4. CONCLUSION

Thus, we have developed an effective and reliable test system for detection of the C/A polymorphism in the bovine kappa-casein *CSN3* gene (rs43703016) by real-time PCR using allele-specific TaqMan fluorescent probes. This method allows genotyping of up to 480 animals (depending on the amplifier model) within 1 hour, and may be used for selection of cows carrying the CC (or BB) genotype, which determines good cheese-making properties of milk.

ETHICAL APPROVAL

As per international standard ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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