

**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, rs43703016, genotyping, real-time PCR

1. INTRODUCTION

Marker-target cattle selection 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; 4 of them affect the characteristics of milk (rs43703015, rs43703016, rs43703017, and rs43706475). 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.

This substitution stirred an interest long ago, when the alleles were shown to affect the content of milk proteins and the quality of cheese [2–5], and this interest is still displayed [6–12]. 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 [13]. 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

36 assessment based on the melting temperature of PCR products of the CSN3 and LGB
37 genes [patent CZ303083B6, 2012], allele-specific PCR [patent RU2013106990A, 2014], and
38 real-time PCR for allele testing of 3 substitutions in the gene at the same time [patent
39 RU2646140C1, 2018]. The latter test system is the most effective one; however, it does not
40 allow assessment of the cis-trans positions of the substitutions in question. As can be
41 concluded, the interest in the development of methods that can be used for allele testing of
42 the rs43703016 substitution in the CSN3 gene is not waning. A simple, fast, and inexpensive
43 method for mass genotyping for this polymorphism in cattle is needed.
44 The objective of the reported study was to develop and verify a method to differentiate A/C
45 alleles of the rs43703016 substitution in the bovine CSN3 gene by real-time PCR (RT-PCR).
46

47 **2. MATERIAL AND METHODS**

48
49 The study was conducted on 94 samples obtained from black-and-white Holsteinized cows
50 of the Pravda-N LLC animal farm (Dzerzhinsk District, Kaluga Region).

51 **2.1 DNA isolation and oligonucleotide design**

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

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

57 **2.2 Real-time PCR**

58 The amplification reaction was performed in 10 μ L of a PCR mixture containing 5 μ L of the
59 LightCycler® 480 Probes Master reagent (Roche, Switzerland), a mixture of the straight
60 primer k-casein-F: 5'-CCATCGAAGCAGTAGAGAGCA-3' and the reverse primer k-casein-
61 R: 5'-GGACTGTGTTGATCTCAGGTGG-3' (10 μ M), 0.4 μ M of each of the allele-specific
62 probes, k-casein-C: 5'-FAM-CTACTCTAGAAGCTTCTCCAG-BHQ1-3' and k-casein-A: 5'-
63 VIC-CTACTCTAGAAGATTCTCCA-BHQ1-3', and 10 ng of the DNA. PCR was carried out
64 using a LightCycler 96 device (Roche, Switzerland) under optimized conditions (95 °C - 10
65 min; 95°C - 20 s, 55°C - 30 s, 72°C - 20 s, 40 cycles). Fluorescence was detected at the
66 elongation stage in the FAM and VIC channels. Results were analyzed with the LightCycler®
67 96 version SW1.1 amplifier software (Roche, Switzerland).

68 **2.3 Method validation**

69 Validation was performed with PCR-RFLP. The PCR utilized 0.2 μ M portions of the primers
70 k-cas-D: 5'-ATAGCCAAATATATCCCAATTCAG-3' and k-cas-R: 5'-
71 TTTATTAATAAGTCCATGAATCTT-3', Fusion Hot Start II High-Fidelity DNA polymerase kit
72 (Thermo Scientific, USA), used in accordance with the manufacturer's instructions and 30 ng
73 of the genomic DNA. The PCR was performed using a T100 amplifier (Bio-Rad, USA) under
74 the following conditions: pre-denaturation at 98°C for 30 s, 40 amplification cycles at - 98°C -
75 10 s, at 55°C - 30 s, at 72°C - 1 min. The restriction was performed in 20 μ L of a mixture
76 containing 2 μ L of a 10x buffer and 1 unit of Hind III endonuclease (Thermo Scientific, USA);
77 obtained amplicons were processed for 16 hours at 37°C. The restriction results were
78 assessed using electrophoresis in 1.2% agarose gel.

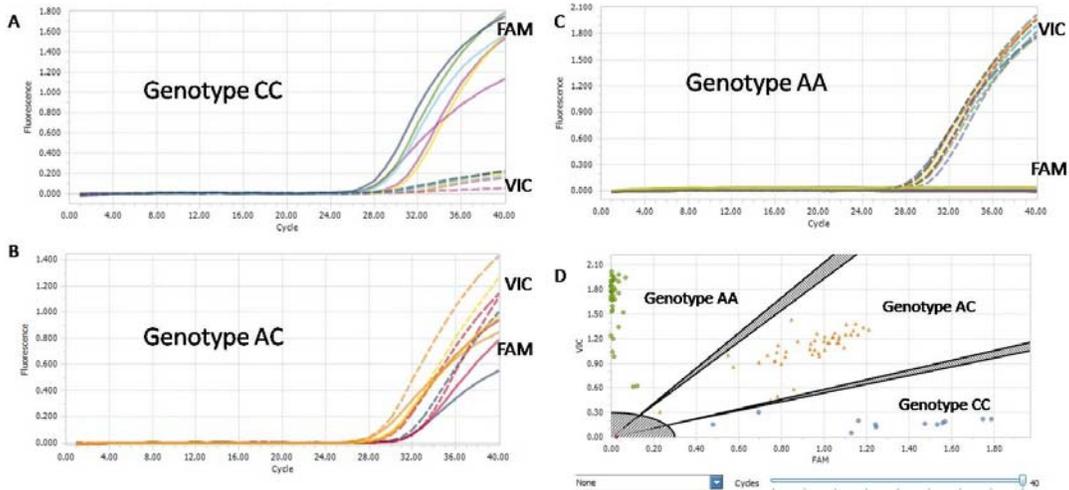
79 The occurrence rates of identified genotypes were calculated by direct counting.
80

81 **3. RESULTS AND DISCUSSION**

82
83 In this study, we developed a new test system for genotyping rs43703016 single-nucleotide
84 substitutions in exon 4 of the kappa-casein gene (CSN3) using real-time PCR. Bovine CSN3
85 was tested using allele-specific fluorescent probes.

86 The developed test system utilized two primers common to both alleles of the CSN3 gene
87 and two allele-specific TaqMan probes labeled with FAM and VIC reporter dyes. The k-cas-
88 D and k-cas-R primers are used to amplify an 85 bp fragment of the CSN3 gene.

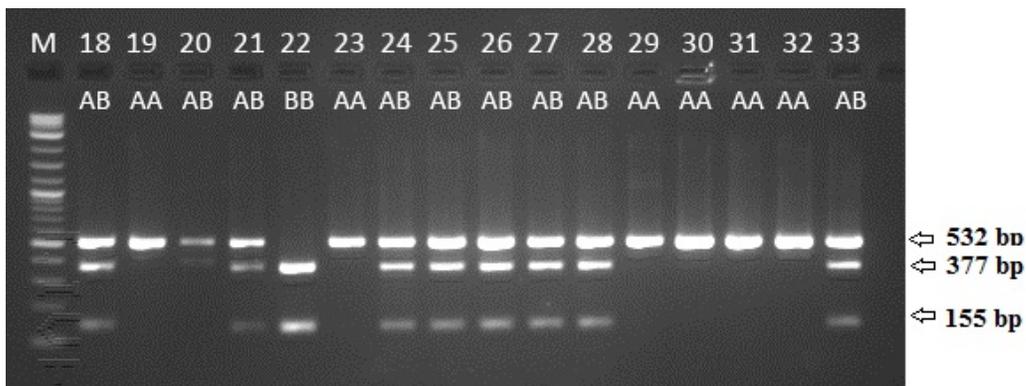
89 Identification of alleles C and A of the rs43703016 substitution in the CSN3 gene is based on
 90 comparison of the fluorescence intensities of the FAM and VIC reporter dyes, respectively.
 91 The genotyping analysis was carried out using the LightCycler® 96 version SW1.1. amplifier
 92 software (Figure 1).
 93



94
 95 **Fig. 1. Example of the detection of allele variants C and A of the rs43703016**
 96 **substitution in the bovine CSN3 gene by real-time PCR. Fluorescence curves (A-C)**
 97 **and the genotype distribution (D) are presented.**
 98

99 CC cows were found to have an increase in fluorescence signal in the FAM channel (Figure
 100 1A). In AA cattle, the fluorescence signal was registered in the VIC channel (Figure 1C).
 101 Heterozygous cows (genotype AC) had signal detected in both channels, FAM and VIC
 102 (Figure 1B). Therefore, RT-PCR results obtained with allele-specific TaqMan probes allow
 103 detection of the presence in the analyzed DNA sample of each of the studied alleles (C and
 104 A) of the rs43703016 substitution in the CSN3 gene and, accordingly, determination of the
 105 animal's genotype.

106 The proposed real-time PCR-based test system was validated on 94 DNA samples obtained
 107 from black-and-white Holsteinized cattle. The genotyping results revealed that 49% of the
 108 animals were carriers of both alleles (genotype CA), 38 % of the cows were allele A
 109 homozygotes (genotype AA), and 13% of the cattle were allele C homozygotes (genotype
 110 CC). These results are generally consistent with available literature data (e.g., [14]).
 111



112
 113 **Fig. 2. An example of cattle genotyping results with regard to alleles A/C of the**
 114 **rs43703016 substitution in the CSN3 gene.**

115
116 The proposed method was validated using PCR-RFLP analysis [15]. Results obtained with
117 the two genotyping methods completely coincided; however, the method proposed in this
118 paper allows significant (up to 1 hour) reduction of the assay time, which gives it an
119 advantage over PCR-RFLP analysis. Additionally, the use of RT-PCR allows to avoid an
120 electrophoresis stage and, consequently, contamination of the laboratory with amplification
121 products of the *CSN3* gene fragment.
122

123 **4. CONCLUSION**

124
125 Thus, we have developed an effective and reliable test system for detection of the C/A
126 polymorphism in the bovine kappa-casein *CSN3* gene (rs43703016) by real-time PCR using
127 allele-specific TaqMan fluorescent probes. This method allows genotyping of up to 480
128 animals (depending on the amplifier model) within 1 hour, and may be used for selection of
129 cows carrying the CC (or BB) genotype, which determines good cheese-making properties
130 of milk.
131

132 **ETHICAL APPROVAL**

133
134 As per international standard ethical approval has been collected and preserved by the
135 authors.
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137 **COMPETING INTERESTS**

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139 Authors have declared that no competing interests exist.
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