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Development of PCR Methods for Cattle Genotyping by Allelic Variants of *DGAT1* Gene.

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ABSTRACT

The main purpose of the research was to develop and prove effective PCR methods for cattle genotyping by allelic variants of the Diacylglycerol O-Acyltransferase 1 (*DGAT1*) gene. The article describes two PCR methods for genotyping of cattle by alleles *A* and *K* of gene *DGAT1*: the first method based on electrophoretic detection, which comprises using two forward allele-specific primers and one reverse primer; and the second method based on real-time hybridization fluorescence detection, which uses two 5′-fluorescently labeled forward allele-specific primers, one non-modified reverse primer, and one anti-primer labeled with fluorescence extinguisher at 3′-end of oligonucleotide. The approved PCR methods have correctly identified the analyzed genotypes, showing consistent results, comparable to the estimated data and to the results of preliminary research studies based on PCR-RFLP of the same cattle sampling.

Keywords: *DGAT1*, allele, genotype, cattle, genotyping, PCR.

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INTRODUCTION

One of the economically valuable genes of the lipid metabolism in cattle is Diacylglycerol O-Acyltransferase 1 (*DGAT1*) [5, 6].

Recent research has studied the influence of *DGAT1* polymorphism on the milk producing ability of various cattle breeds, with an emphasis of the content and output of milk fat [5, 7, 10, 12], as well as its influence on fat acid, protein, and mineral structure of milk [6].

The most poplar method for evaluation of allele polymorphism of *DGAT1* gene of cattle is the method of various PCR-RFLP modifications [2, 4, 9].

In addition, animal genotyping by this gene includes methods for direct sequencing, high resolution melting curve analysis, (HRM) [3], as well as real-time PCR [13].

Research studies related to developing molecular approaches to *DGAT1* gene's allele polymorphism estimation are relevant and are of scientific practical interest.

The main purpose of the research is to develop and prove effective PCR methods for cattle genotyping by allelic variants of the *DGAT1* gene.

MATERIALS AND METHODS

Extraction of nucleic acid from samples of whole blood of cattle conserved with 10 mM of EDTA-Na2 was provided by the combined alkaline method [1].

The developed PCR methods for genotyping of cattle by alleles *A* and *K* of the *DGAT1* gene were implemented using the Rotor Gene 6000 amplifier (Corbett Research, Australia) in the volume of 20 µl each, containing a standard buffer (60 mM of Tris-HCl (pH 8.5); 1.5 mM of $MgCl_2$; 25 mM of KCl; 10 mM of 2-mercaptoethanol; 0.1% of Trition X-100), 0.25 mM of dNTP, 1 unit of Taq DNA polymerase, 0.25 µM of forward allele-specific primers each (DGAT1A+DGAT1K, DGAT1-A+DGAT1-K), 0.5 µM of reverse universal primers (DGAT1R, DGAT1-R) and anti-primer DGAT1-S each.

A list of used oligonucleotides and the conditions of PCR for identification of alleles *A* and *K* of the *DGAT1* gene of cattle are represented in the same-name table.

Table 1: List of used oligonucleotides and the conditions of PCR to identify alleles *A* and *K* of the *DGAT1* gene of cattle

Names and sequence of oligonucleotides	Amplification modes
DGAT1A: 5′-CGTAGCTTTGGCAGGTAAAGC-3′ (21 n.) DGAT1K: 5′-CCGCTTGCTACTAGCTTTGGCAGGTAACAA-3′ (30 n.) DGAT1R: 5′-TCAGGTTGTCGGGGTAGCTC-3′ (20 n.)	×1: 94°C – 4 min ×40: 94°C – 20 sec, 65°C – 20 sec, 72°C – 20 sec. ×1: 72°C – 5 min.
DGAT1-A: 5′-ROX-CGTAGCTTTGGCAGGTAAAGC-3′ (21 n.) DGAT1-K: 5′-Cy5-CGTAGCTTTGGCAGGTAACAA-3′ (21 n.) DGAT1-R: 5′-GGCAGCTCCCCCGTTGG-3′ (17 n.) DGAT1-S: 5′-ACCTGCCAAAGCTACG-3′-BHQ2 (16 n.)	×1: 94°C – 4 min ×40: 94°C – 10 sec, 50°C – 10 sec, 72°C – 10 sec, 50°C – 10 sec [Orange-ROX/Red-Cy5]

The detection of the result of PCR with primers DGATA, DGATK and DGATR (Table 1) was provided by horizontal electrophoresis in 3% agarose gel in TBE buffer (pH 8.0), containing 0.5 µg/ml concentration of ethidium bromide, followed by further visualization of the amplified products in a UV transilluminator (λ = 310 nm).

DNA was estimated by mobility compared to standard DNA markers.

The hybridization fluorescence detection of the result of PCR with primers DGAT1-A, DGAT1-K, DGAT1-R and anti-primer DGAT1-S (Table 1) was provided on a real-time basis.

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Chemical agents for molecular and biological research produced by LLC SibEnzim (Russia) and LLC DNA-Syntez (Russia) were used in the study.

Approval of the developed PCR methods for cattle genotyping by alleles *A* and *K* of the *DGAT1* gene was provided with DNA samples of 70 well-bred and crossbred Holstein servicing bulls of SUE GPP Elita of the Vysokogorsky District of the Republic of Tatarstan of the Russian Federation.

RESULTS

We have developed a PCR method for genotyping of cattle by alleles *A* and *K* of the *DGAT1*-gene in the form of electrophoretic detection, which provides using two forward allele-specific (DGAT1A+DGAT1K) and one reverse (DGAT1R) primers, generating amplification of corresponding genotype-specific products (*AA* = 71 bp, *KK* = 80 bp, *AK* = 80/71 bp) (Figure 1).

```
DGAT1A:                  5/-CGTAGCTTTGGCAGGTAAAGC-3/
DGAT1K:        5/-CCGCTTGCTACTAGCTTTGGCAGGTAACAA-3/
Allele A: 01             5/-CGTAGCTTTGGCAGGTAAGGCGGCCAACGGGGGAGCTGCCCAGC
Allele K: 01 5/-CCGCTTGCT.....................AA.......................
                                                PCR-product
Allele A: 45 5/-GCACCGTGAGCTACCCCGACAACCTGA-3/ 71 bp
Allele K: 54 5/-............................-3/ 80 bp
                        ||||||||||||||||||||
                     3/-CTCGATGGGGCTGTTGGACT-5/:DGAT1R
```

Figure 1: Alignment of the flanked with primers DGAT1A + DGAT1K + DGAT1R nucleotide sequences of alleles *A* and *K* of the *DGAT1 Bos Taurus* gene

The visual result of approval of the proposed PCR method through electrophoretic detection is presented in Figure 2.

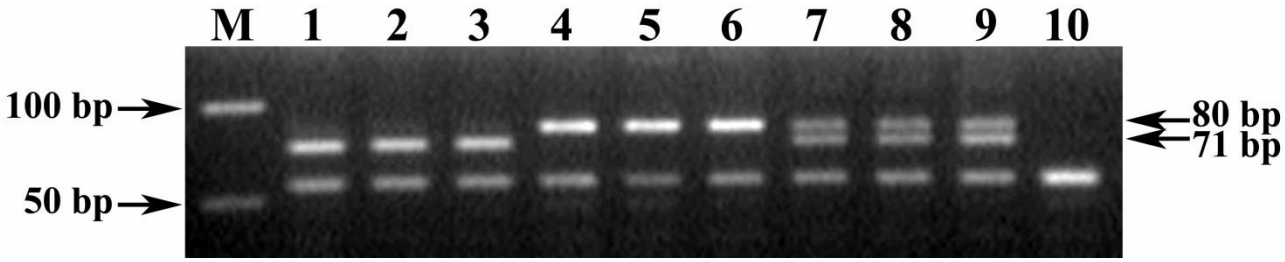

Figure 2: Electrophoretogram of the result of the proposed PCR method for cattle genotyping by alleles *A* and *K* of the *DGAT1* gene.

(primers DGAT1A + DGAT1K + DGAT1R): M) DNA markers 100 bp + 50 bp (SibEnzim). 1-3) genotype *AA* of the *DGAT1* gene of cattle. (71 bp). 4-6) genotype *KK* of the *DGAT1* gene of cattle. (80 bp). 7-9) genotype *AK* of the *DGAT1* gene of cattle. (80/71 bp). 10) NCS (negative control sample).

Thus, as a result of practical studies targeting to prove our developed PCR method for genotyping of cattle by alleles *A* and *K* of the *DGAT1* gene in the form of electrophoretic detection, we obtained a technical result that can be achieved by the proposed method, which ensures effective identification of the sought genotypes (*AA, KK, AK*) due to correct interpretation of the generated genotype-specific PCR products, 80 bp and(or) 71 bp in length.

The primers used in the reaction additionally initiated amplification of a non-specific PCR product, approximately 55 bp in size, which did not validly influence on correct interpretation of the results of genotyping.

We have also developed a PCR method for cattle genotyping by alleles *A* and *K* of the *DGAT1* gene in the form of real-time hybridization fluorescence detection, which assumes using two 5/-fluorescently labeled

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forward allele-specific primers (DGAT1-A+DGAT1-K), one non-modified reverse primer (DGAT1-R), and one anti-primer (DGAT1-S) labeled with fluorescence extinguisher at 3′end of oligonucleotide (Figure 3).

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DGAT1-A:  5′-ROX-CGTAGCTTTGGCAGGTAAAGC-3′                          PCR-
DGAT1-K:  5′-Cy5-CGTAGCTTTGGCAGGTAACAA-3′                          product
Allele A: 01 5′-CGTAGCTTTGGCAGGTAAGGCGGCCAACGGGGGAGCTGCC-3′ 40 bp
Allele K: 01 5′-....................AA....................-3′ 40 bp
Anti-primer      ||||||||||||||||||        ||||||||||||||||||
DGAT1-S:3′-BHQ2-GCATCGAAACCGTCCA-5′  3′-GGTTGCCCCCTCGACGG-5′:DGAT1-R
```

Figure 3: Alignment of the flanked with primers DGAT1-A + DGAT1-K + DGAT1-R nucleotide sequences of alleles *A* and *K* of the *DGAT1 Bos Taurus* gene

The visual result of approval of the proposed real-ime PCR method is presented in Figure 4.

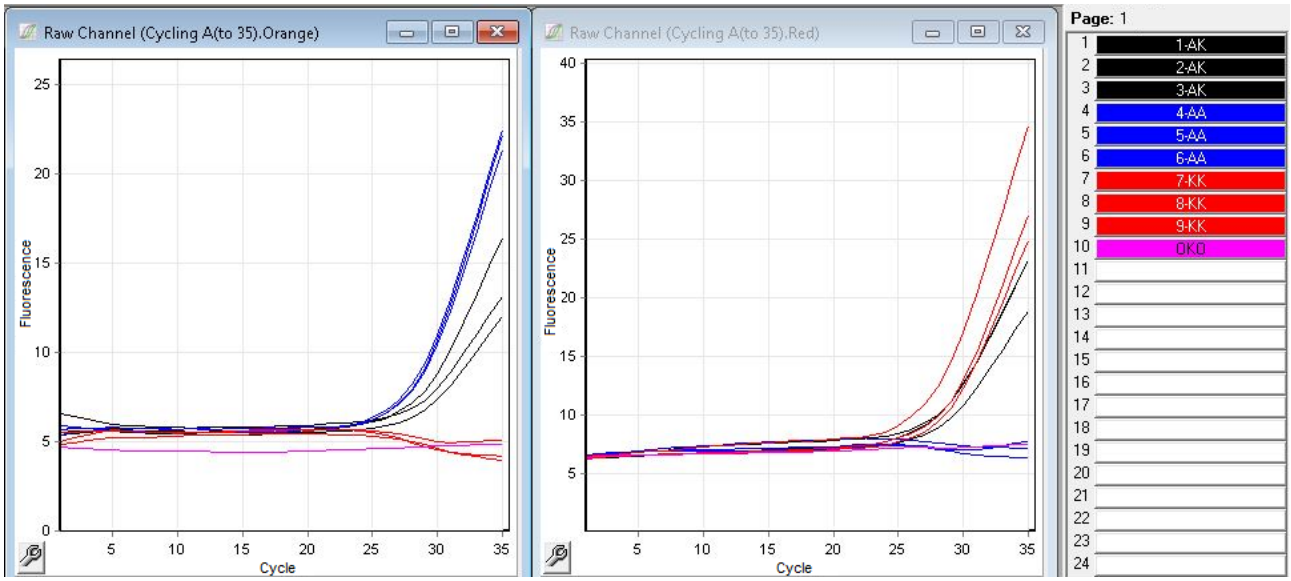

Figure 4: Result of the proposed real-time PCR method for cattle genotyping by alleles *A* and *K* of the *DGAT1* gene (primers DGAT1-A+DGAT1-K+DGAT1-R and anti-primer DGAT1-S): On the left – Orange detection channel. On the right – Red detection channel. Fluorescence increase curves for genotypes *AA* (blue lines), *AK* (black lines) and *KK* (red lines). NCS (pink lines).

As a result of practical studies targeting to prove our developed PCR method for genotyping of cattle by alleles *A* and *K* of the *DGAT1*-gene in the form of real-time hybridization fluorescence detection, we additionally obtained a technical result that can be achieved by the proposed method, which enables effective identification of the sought genotypes (*AA, KK, AK*) due to correct interpretation of the fluorescence intensity increase curves' data.

DISCUSSION

Our developed PCR method for cattle genotyping by alleles *A* and *K* of the *DGAT1* gene in the form of electrophoretic detection in agarose gel is a variety of allele-specific PCR (AS-PCR), proposed by M. Gaudet et al. (2009) [8].

This variety of allele-specific PCR enables effective discrimination of single nucleotide polymorphisms (SNPs) in standard conditions of a single reaction, using two forward allele-specific primers of different length (with 3′-end bases, complementary to SNP-site) and one universal reverse primer. The selected combination of primers initiates amplification of allele-specific PCR of various length products, divided further by agarose gel-electrophoresis [8].

In addition, this variety of AS-PCR [8] provides addition of the destabilizing mismatch-nucleotide into 3′-end segment of each allele-specific primer, in particular, located in the oligonucleotides DGAT1A and DGAT1K built by us in position –2 (mismatched nucleotide in 3-rd position from 3′-end of the primer). In this case we additionally added two more mismatched nucleotides in the DGAT1K primer, extended by 9 bases, into positions –19 and –20, to increase the specificity of the reaction.

Another our developed PCR method for genotyping of cattle by alleles *A* and *K* of the *DGAT1* gene in the form of hybridization fluorescence detection is related to the category of anti-primer-based quantitative real-time PCR (aQRT-PCR), proposed by J. Li & G.M. Makrigiorgos (2009) [11], which is also capable of effective genotyping of biological objects based on the polymorphism of single nucleotic substitutions.

The main difference of our developed method from prototypes [11, 13] resides in the peculiar features of 5′-fluorescently labeled forward allele-specific primers that completely consist of gene-specific sequences, on which, including by means of concurrent hybridization, the complementary anti-primer of shorter length is burnt, which is labeled by the fluorescence extinguisher on the 3′end of oligonucleotide.
The proven in this article PCR methods for cattle genotyping by alleles *A* and *K* of the *DGAT1* gene correctly identified the genotypes under analysis, providing a consistent result comparable to the estimated data, including the data obtained as a result of an analysis of alignment of nucleotide sequences flanked by respective primers (Figure 1, Figure 3), and also comparable with the data of previous studies based on PCR-RFLP of the same sampling of cattle [2].

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