APPROBATION OF PCR-RFLP AND AS-PCR METHODS FOR GENOTYPING CATTLE BY THE DGAT1 GENE

Abstract. The DGAT1 gene Bos taurus is an economically valuable lipid metabolism gene in cattle, affecting upon milk production and milk quality, the allele polymorphism assessment of which is diagnostically significant. The purpose of this work was to test a number of methods for carrying out PCR-RFLP and AS-PCR for genotyping cattle using allelic variants of the DGAT1 gene and then evaluating allelic polymorphism in the studied animal sample in the context of consistency of the used molecular genetic approaches. Studies have been conducted on a sample of mixed and pure bred Holstein cattle. DNA Extraction from samples of whole canned blood of cattle carried out by the combined alkaline method. Proven methods of genotyping effectively identified the analyzed genotypes, showing consistent with each other reliable results, in accordance with the calculated data of alignments analysis, restriction mapping and simulations of generated PCR and RFLP profiles.

Keywords: Bos taurus, DGAT1, allele: genotype, genotyping, PCR, RFLP.

Introduction. PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) – combined molecular genetic method for genotyping animals [1], plants [2], bacteria [3] and viruses [4], where the length of the generated amplicons and restricts is analyzed for single nucleotide polymorphism (SNP) and allelic polymorphism in eukaryotes, in particular, with the subsequent conclusion about the homozygous or heterozygous state of a certain gene of the individual being studied.

Various modifications of PCR-RFLP analysis [5-7] have found practical application in evaluating the allelic polymorphism of DGAT1 (diacylglycerol o-acyltransferase 1) Bos taurus, an economic-valuable lipid metabolism gene in cattle [8, 9], affecting on milk production and milk quality in the context of the content and yield of milk fat [10-12], as well as fat-acid, protein and mineral composition of milk [9], which is especially important in the production of functional and gerodietic dairy products [13-15, 21].

Along with this, other of SNP detection methods, such as AS-PCR (Allele-Specific PCR) [16], direct sequencing of the amplified gene locus, HRM analysis (High Resolution Melting) [17] and Real-time PCR [16, 18].

The goal of the study was to test a number of PCR-RFLP and AS-PCR methods for genotyping cattle using allelic variants of the DGAT1 gene and then evaluating allelic polymorphism in the studied animal sample in the context of consistency of the molecular used genetic approaches.

Materials and Methods. Studies were conducted on a sample of mixed and pure bred Holstein cattle of one of the breeding enterprises of the Russian Federation.

Extraction of nucleic acid from samples of whole blood of cattle, canned 10 mm EDTA-Na2, carried out by the combined alkaline method [19].

Tested methods of PCR-RFLP and AS-PCR for cattle genotyping for alleles A and K of the DGAT1 gene were performed on a "Tertsik" amplifier ("DNA -Technology, Russia) in volumes of 20 µl,
containing standard buffer (60 mM Tris -HCl (pH 8.5); 1.5 mM MgCl2; 25 mM KCl; 10 mM 2-mercaptoethanol; 0.1% Triton X-100), 0.25 mM dNTP, 1 U Taq DNA polymerase, 0.25 mM of direct standard (DGAT1-F [19]) and allele-specific primers (DGAT1-1 + DGAT1-2 [7] and DGAT1A + + DGAT1K [16]), 0.5 µM of reverse common primers (DGAT1-R [19], DGAT1-3 [7] and DGAT1IR [16]), 1 µl of the DNA sample in the following thermal cycling modes:

- PCR with primers DGAT1-F + DGAT1-R:
  

- PCR with primers DGAT1-1 + DGAT1-2 + DGAT1-3:
  
  ×1: 94°C – 4 min; ×40: 94°C – 10 sec, 72°C – 10 sec.; ×1: 72°C – 5 min; storage: 4°C.

- PCR with primers DGAT1A + DGAT1K + DGAT1R:
  

The sequence of used oligonucleotide primers sets is presented in table 1.

<table>
<thead>
<tr>
<th>Oligonucleotide Primers Sets for PCR-RFLP and AS-PCR</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGAT1-F: 5′-GCACCATCCTCTCTTCCAAG-3′</td>
<td>[19]</td>
</tr>
<tr>
<td>DGAT1-R: 5′-GGAAGGCCTTTCGGAATG-3′</td>
<td></td>
</tr>
<tr>
<td>DGAT1-1: 5′-CGGTCTGTACGCTTCCAGGTAAGG-3′</td>
<td>[7]</td>
</tr>
<tr>
<td>DGAT1-2: 5′-CGGTCTGTACGCTTCCAGGTAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>DGAT1-3: 5′-CGGTCTGTACGCTTCCAGGTAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>DGAT1A: 5′-CGTCTGTTGGCAGTTAAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>DGAT1K: 5′-CGTCTGTTGGCAGTTAAAGG-3′</td>
<td></td>
</tr>
<tr>
<td>DGAT1R: 5′-TCAGTTGAGGTCTTATG-3′</td>
<td>[16]</td>
</tr>
</tbody>
</table>

To determine the allelic polymorphism of the DGAT1 gene with use of allelic variants A and K, 20 µl of PCR samples were treated with 10 U of AcoI restrictee in 1× buffer “G" at 37°C (DGAT1-F + +DGAT1-R) and 20 U of TaqI restriction in 1× buffer "Y" (DGAT1-1 + DGAT1-2 + DGAT1-3) at 65°C overnight.

The results of PCR-RFLP and AS-PCR were detected by horizontal electrophoresis in 3% agarose gel in TBE buffer (pH 8.0), containing ethidium bromide at a concentration of 0.5 µg/ml, followed by visualization of the amplified products in an ultraviolet transilluminator (λ = 310 nm).

We used reagents for molecular biological studies produced by SibEnzyme LLC (Russia) and DNA-Synthesis LLC (Russia).

\[ p = \frac{n}{N}, \]

where \( p \) - genotypes frequency; \( n \) - number of animals having a certain genotype; \( N \) - total number of examined animal units.

The calculation of individual alleles frequency is determined by the formula:

\[ p = \frac{(2N1+N2)/2n}, \]

where \( N1 \) - homozygotes number for studied allele; \( N2 \) - heterozygotes number; \( n \) - sample size.

To compare the observed and expected frequency distribution of genotypes, the chi-square correspondence criterion is used (\( \chi^2 \)).

The expected frequencies in the studied sample are calculated according to the Hardy-Weinberg law.

The obtained results were processed by a biometric method using computer and Microsoft Excel software application. The level of their reliability is determined by the Student criterion.

Results and discussion. Calculated data for verification of PCR-RFLP and AS-PCR validated methods were obtained based on alignment analysis, AcoI and TaqI restriction mapping of amplified partial nucleotide sequences of A and K allelic variants DGAT1 Bos taurus gene with modeling of the generated PCR and RFLP profiles of the corresponding genotypes.

Thus, the well-known primers DGAT1-F and DGAT1-R [19] initiate amplification of the cattle DGAT1 gene locus with a length of 411 bp, and AcoI-RFLP analysis of the genotype-specific fragments generated (AA = 208/203 bp, KK = 411 bp and AK = 411/208/203 bp) provides the correct genotyping procedure (table 2).
Another tested primer set DGAT1-1 + DGAT1-2 + DGAT1-3 [7] triggers the amplification of DGAT1 Bos taurus gene locus with a length of 100 bp followed by the generation of DGAT1-PDL-P-TaqI profiles (AA = 82/18 bp, KK = 100 bp and AK = 100/82/18 bp) (table 3).

The result of modeling the generated PCR and TaqI-RFLP profiles of the corresponding genotypes for the DGAT1 gene is presented in figure 1.

An illustrative result of PCR-RFLP for genotyping cattle by alleles A and K of the DGAT1 gene with the corresponding primers (DGAT1-1 + DGAT1-2 + DGAT1-3) and endonuclease digestion with the restriction enzyme TaqI is presented in figure 2.

At the same time, a distinctive feature of this approach is that at the PCR stage, the “Single PCR” formulation uses three primers, one of which (DGAT1-3) is common for both alleles of the analyzed gene, and the other two (DGAT1-1 + DGAT1-2) - allele-specific, but with a given restriction identification site for one of them (DGAT1-1), and artificially created, but not affecting the analyzed SNP itself, which is a competitive advantage when choosing the desired restriction enzyme (figure 3).
Fig. 2 Electrophorogram of the Result of a PCR-RFLP with the Primers DGAT1-1 + DGAT1-2 + DGAT1-3 and endonuclease digestion with the restriction enzyme TaqI for genotyping Bos taurus on the Allelic Variants A and K of the DGAT1 gene.

Note: M) DNA Molecular Weight Marker (100 bp + 50 bp ladder); 1, 3, 5, 6) genotype AK (100/82/18 bp); 2) genotype AA (82/18 bp); 4) genotype KK (100 bp).

Figure 3 – Aligning and Tag-Restriction Mapping of the Nucleotide Sequence of DGAT1-gene locus of the Bos taurus Flanked with Primers DGAT1-1 + DGAT1-2 + DGAT1-3 (Alleles A and K)

The third tested primer set DGAT1A + DGAT1K + DGAT1R [16] also provides effective identification of the desired genotypes (AA, KK, AK) due to the correct interpretation of the generated 80-bp and/or 71 bp genotypes (table 4).

Table 4 – Oligonucleotide Primers Third Set for AS-PCR

<table>
<thead>
<tr>
<th>Oligonucleotide Primers Set</th>
<th>PCR Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGAT1A + DGAT1K + DGAT1R</td>
<td></td>
</tr>
</tbody>
</table>

An illustrative electrophoretic picture of the AS-PCR result for the genotyping of cattle by alleles A and K of the DGAT1 gene with a set of oligonucleotide primers DGAT1A + DGAT1K + DGAT1R is shown in figure 4.

Figure 4 – Electrophorogram of the Result of a AS-PCR with the Primers DGAT1A + DGAT1K + DGAT1R for Genotyping Bos taurus on the Allelic Variants A and K of the DGAT1 gene.

Note: M) DNA Molecular Weight Marker (100 bp + 50 bp ladder); 1-2) genotype AA (71 bp); 3-4) genotype KK (80 bp); 5-6) genotype AK (80/71 bp).
This method of carrying out PCR for genotyping cattle in allelic variants A and K of the DGAT1 gene with detection in agarose gel electrophoresis is a type of allele-specific PCR (AS-PCR) [20].

It effectively discriminates against single nucleotide substitutions (SNPs) in the “Single PCR” formulation when using two forward allele-specific primers of different lengths with 3/- end bases complementary to the SNP site (DGAT1A + DGAT1K and one common reverse primer (DGAT1R), together initiating amplification of allele-specific PCR products of various lengths, separated in agarose gel electrophoresis (figure 5).

<table>
<thead>
<tr>
<th>DGAT1-gene</th>
<th>CGTACCTTGGGCAGGTAAAGC = A-allele-specific primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele A</td>
<td>01 CAGTAGCTTGTGCCAGGTAAACAA = K-allele-specific primer</td>
</tr>
<tr>
<td>Allele K</td>
<td>01 CCGCTTGGCT..................AA....................</td>
</tr>
</tbody>
</table>

Common primer PCR-
DGAT1-gene
GAGCTACCCGACAACCTGA product
Allele A 45 GCACCGTGAGCTACCCGACAACCTGA 71 bp
Allele K 54 ........................................ 80 bp

Figure 5 - Aligning of the Nucleotide Sequence of DGAT1 gene locus of the Bos taurus Flanked with Primers DGAT1A + DGAT1K + DGAT1R (Alleles A and K)

At the same time, to increase the specificity of the reaction, an unpaired nucleotide in the 3rd position from the 3/- end of oligonucleotides is introduced into the allele-specific primers, as well as two additional mismatch nucleotides in the 20th and 21st positions only in the DGATK primer.

The approved genotyping methods showed identical results of the evaluation of the allelic polymorphism of the DGAT1 gene in the sample of manufacturing bulls, whose data are presented in Table 5.

Table 5 –Allele polymorphism of the DGAT1 gene in the studied sample of servicing bulls

<table>
<thead>
<tr>
<th>N=70</th>
<th>Occurrence Frequency</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>genotypes</td>
</tr>
<tr>
<td></td>
<td>AA</td>
</tr>
<tr>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>O</td>
<td>38</td>
</tr>
<tr>
<td>E</td>
<td>38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>alleles</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.74</td>
</tr>
<tr>
<td>K</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
</tr>
</tbody>
</table>

Note: O – actually observed indicator, E – theoretically expected indicator.

So, bulls, which are mixed and pure bred Holstein cattle, the frequency of allele A was 0.74, allele B - 0.26, and genotype distribution was as follows: AA - 38 heads (54.3%), AK - 28 heads (40.0%), KK - 4 heads (5.7%).

The observed frequency distribution of genotypes in the studied sample corresponds to the theoretically expected Hardy-Weinberg equilibrium distribution.

Conclusion. PCR-RFLP and AS-PCR methods for genotyping cattle using allelic variants A and K of the DGAT1 gene effectively identified the analyzed genotypes, showing reliable results consistent with each other, in accordance with the calculated data of alignment analysis, restriction mappings and modeling of the generated profiles, comparable with the results of a previous study on the same sample of manufacturing bulls [7].
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DGAT1 ГЕНИ БОЙЫНША ИРИ КАРА ЖАНУАРЛЫРЫН ГЕНОТИПТЕНДІРУ УШЫН PCR-RFLP ЖӘНЕ AS-PCR ЭДІСТЕРІНІҢ СЫНАҚТАН ОТКУЗУ

Аннотация. DGAT1 Bos taurus – бұл маңызды сутилігіне және сапасына есер ететін, аалдұлай полиморфизмді балықта да диагностикалық мәні бар ірі кара жануарының май алаңына қатыстыны мәңдегі ген. Осы жұмыстарды мәсете – DGAT1 генінің аалдұлай нәсілдарын қолдану арқылы PCR-RFLP және AS-PCR операцияларының жүргізу үшін біркәтар эдистері сияқты отқызу, содан кейін зерттелген жануарлар ұлғыс ісінде қолданылатын молекулярлық ғылымдық тәсілдердің ұысымысы әлдеқайсы аалдұлай полиморфизмді балықта. Жүргізулердің жұмысы және тәріздеме құрайтын дәлелгеу әсерінен дәлелдегі тақырып және сүйкес PCР және RFLP профілерінің генерациялайтын әдеттегі картаға ұатұрылған әлдеқайсы, бір-біріне сәйкес болуы әлдеқайсы қатарын сақтайды.

Түйін сөздер: Bos taurus, DGAT1, аалдұлай: генотип, генотиптеге, TP, RFLP.

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АПРОБАЦИЯ СПОСОБОВ ПРОВЕДЕНИЯ PCR-RFLP И AS-PCR ДЛЯ ГЕНОТИПИРОВАНИЯ КРУПНОГО РОГАТОГО СКОТА ПО ГЕНУ DGAT1

Аннотация. Ген DGAT1 Bos taurus – хозяйствен-ценный ген липидного обмена у крупного рогатого скота, влияющий на молочную продуктивность и качество молока, оценку аалдұлай полиморфизм ая со- рого, диагностического значения. Из работы было видно, что аалдұлай полиморфизм DGAT1 с последующей оценкой аалдұлай полиморфизм у исследуемой выборки животных в контексте согласованности используемых молекулярно-генетических подходов. Исследования проведены на выборке племенного и чистопородного говяжьего скота. Экстракция ДНК из образцов цельной консервированной крови крупного рогатого скота осуществлена комбинированным щелочным способом. Апробированные способы генотипирования эффективно идентифицировать генотипы, показав согласованные результаты, в соответствии с расчетными данными анализа выравниваний, репликативных копирований и моделированный генерируемы PCR и RFLP профилей.

Ключевые слова: Bos taurus, DGAT1, аалдұлай: генотип, генотипирование, ПЦР, RFLP.

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REFERENCES


