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Development of the Real-Time PCR method for detection of ss52050737 polymorphism of *lhcgr* gene in cattle

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***Abstract*—Embryo transfer is one of the key technique in reproductive animal biotechnology. One of its crucial step is the selection of donor cows that are most sensitive to the hormonal stimulation of superovulation. One of the promising genetic markers of the reproductive status of cattle is the gene encoding the luteinizing hormone/choriogonadotropin receptor (LHCGR). One of the single nucleotide polymorphisms in the *lhcgr* gene of cattle – 1401G>T (ss52050737) – was shown to be associated with superovulation traits, the number of oocytes and the quality of embryos. To date, PCR-RFLP is one of the most common methods for detection of genes polymorphisms. But the main disadvantages of the PCR-RFLP method are long duration and laboriousness of the analysis. The real-time PCR method with allele-specific hydrolysis probes (TaqMan) is an effective alternative to PCR-RFLP analysis. The aim of this work was to develop the method for detection of ss52050737 polymorphism of *lhcgr* gene in cattle based on real-time PCR. In the developed method, two primers flaking the 113-bp fragment of *lhcgr* gene, and two allele-specific TaqMan probes were used. The identification of 1401G/T alleles (ss52050737 polymorphism) was based on the comparison of VIC and FAM fluorescence, respectively. The developed method was validated by PCR-RFLP analysis on 195 samples of cows of Russian Black Pied cattle. The developed method allows genotyping up to 480 animals (depending on the model of a thermal cycler) for 1 hour and can be used in selection of donor cows with superovulatory potential.**

Keywords—LHCGR; ss52050737 polymorphism; cattle; real-time PCR; TaqMan

I. INTRODUCTION

Development of embryo transfer technology began about a hundred years ago, and in the 1970s it was applied in the commercial field. In 2016, for example, 632,638 embryos were received from 93,815 donor cows, of which 196,000 were transferred immediately, and the remaining were cryopreserved [1, 2]. For many years, North America was the leader in the field of embryo transfer – 52.5% of the total number of embryos *in vivo* was obtained in USA and Canada, while in Europe – only 20.4% [1, 2]. In connection with a wide distribution of embryo transfer technology in cattle reproduction, there is a need to search a reliable genetic marker for selection of donor cows that are sensitive to the hormonal stimulation of superovulation.

The induction of superovulation is based on the administration of gonadotropins to animals, one of which is the luteinizing hormone [3]. The cellular actions of luteinizing hormone are mediated by the luteinizing hormone/choriogonadotropin receptor (LHCGR). LHCGR belongs to the class of membrane receptors associated with G-proteins [4]. In cattle, the *lhcgr* gene is located on chromosome 11 and consists of 11 exons [5]. The *lhcgr* gene is considered as a candidate gene for predicting a response to the induction of superovulation, and the polymorphic variants identified in the *lhcgr* gene was considered to be associated with the cattle fertility and the superovulation potential of the cows [6–11]. The most significant polymorphic variants are non-synonymous single nucleotide polymorphisms (SNPs) located in exons of the genes, which lead to the replacement of amino acids and affect the proteins functions. The 401G>T replacement (ss52050737 polymorphism) in the *lhcgr* gene was found to be potential marker for superovulation traits. It was shown that cows with GG or GT genotypes were characterized by higher number of oocytes as well as embryos survived after transplantation. Besides, cows with the GG genotype have the least number of unfertilized oocytes compared with the cows with other two genotypes [8].

In the current study, we aimed to develop a reliable and rapid method for detection of ss52050737 polymorphism of *lhcgr* gene in cattle based on real- time PCR.

II. MATERIALS AND METHODS

A. DNA isolation and oligonucleotides design

The study was conducted using 195 blood samples from cows of Russian Black Pied cattle. The genomic DNA was isolated from the whole blood using the *M-sorb* kit (Syntol, Russia) and the protocol provided by the manufacturer.

The GeneRunner and Multiple Primer Analyzer software programs were applied for the design of primers

and TaqMan probes. Primers and probes were synthesized by "DNA-synthesis LLC» (Moscow, Russia).

B. Real time-PCR

The real-time PCR was performed in 10 µl of reaction mix contained 5 µl of LightCycler®480 Probes Master reagent (Roche, Switzerland), forward primer lhcgr-F: 5'-TGAACTCTCTGTCTACAC CCTCACA-3' (0.4 µM), reverse primer lhcgr-R: 5'-GCATGACTGGAATGGCATGTT-3' (0.4 µM), and allele-specific fluorescent probes lhcgr-T: 5'-(FAM)-CACTAGAAAGATGTCACACC-(BHQ1)-3' (0.2 µM) and lhcgr-G: 5'-(VIC)-CTAGAAAGATGGCACACC-(BHQ1)-3' (0.2 µM). The amount of DNA was 10 ng per reaction.

The real-time PCR was run on LightCycler® 96 (Roche, Switzerland) under optimized conditions (95 °C for 10 min; 95 °C for 15 sec, 58 °C for 30 sec, 72 °C for 20 sec, 40 cycles). Fuorescence signals were detected at the elongation step on the FAM and VIC channels. The results of genotyping were analyzed using the LightCycler® 96 thermal cycler software (version SW1.1).

C. Validation of the method

The validation of the developed method was conducted by PCR-RFLP method. PCR mixture of 20 µl contained 1×buffer with 3 mM $MgCl_2$ and 1 unit of HS Taq DNA polymerase (Evrogen, Russia), 0.2 µM of lhcgr_RFLP-Dir: 5'-ACAGTCCCCCGCTTTCT-

CAT-3' and 0.2 µM of RFLP-Rev: 5'-TGACACCCACAAGAGGCAAC-3' primers, 10 ng DNA. PCR was run on LightCycler® 96 (Roche, Switzerland) under conditions: 95 °C for 15 sec.; 95 °C for 15 sec., 62 °C for 15 sec., 72 °C for 15 sec. (40 cycles).

Restriction was performed in 20 µl of reaction mixture contained 2 µl of 10x buffer G and 1.5 µl of NmuCI (Thermo Scientific, USA) during 16 h at 37°C. Restriction results were evaluated by electrophoresis in 1,2% agarose gel.

The frequency of occurrence of the identified genotypes was calculated by direct counting. The allele frequencies were calculated using the formula:

$$P(A) = \frac{(2N_1+N_2)}{2n}, \quad (1)$$

where N1 – the number of homozygotes for the allele studied; N2 – number of heterozygotes; n – sample size.

III. Results and Discussion

To date, one of the most common methods for identification of allelic variants of genes is PCR-RFLP analysis. The PCR-RFLP is a multistep method consisting of i) a genomic DNA isolation; ii) amplification of the polymorphic region of a gene; iii) restriction of the obtained amplicon by the endonuclease (restrictase); iv) electrophoretic separation and analysis of the obtained DNA fragments. The main disadvantages of the PCR-RFLP method include long duration, laboriousness and low productivity of the analysis, as well as unreliable results in cases of non-optimal DNA : restrictase ration and duration of the restriction reaction [12, 13].

The real-time PCR method with allele-specific hydrolysis probes (TaqMan) is an effective alternative to PCR-RFLP. In the present work we developed a real-time PCR method for detection of ss52050737 polymorphism of *lhcgr* gene in cattle. In the developed method we used two primers common for the both *lhcgr* gene alleles, and two allele-specific TaqMan probes. The lhcgr-F and lhcgr-R primers initiate the amplification of 113-bp fragment of the *lhcgr* gene. The identification of 1401G>T alleles (ss52050737 polymorphism) is based on the comparison of fluorescence intensities of VIC and FAM dyes, respectively. The genotyping analysis was performed by the LightCycler® 96 thermal cycler software (version SW1.1) (Fig. 1).

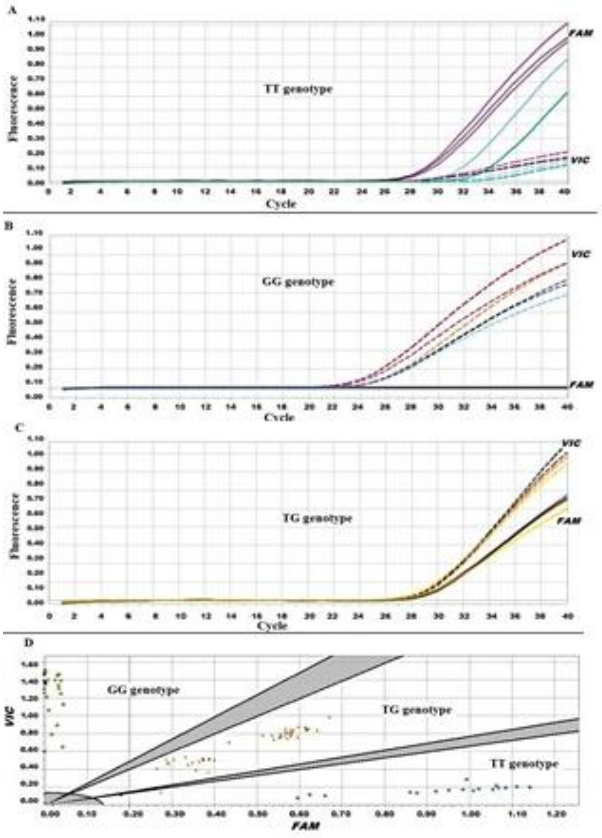

Fig. 1. The example of detection of 1401G>T alleles (ss52050737 polymorphism) of lhcgr gene in cattle by real-time PCR method. Fluorescence curves (A–C) and distribution diagram for all genotypes (D) are shown. Analysis was performed by the LightCycler® 96 thermal cycler software (version SW1.1).

For cows with TT genotype, the increase in fluorescence is detected on FAM channel (Fig. 1A). For cows with GG genotype the fluorescent signal is registered on VIC channel (Fig. 1B). In the case of heterozygous cows (TG genotype) the fluorescent signals are observed on both FAM and VIC channels (Fig. 1C). Thus, the results of the real-time PCR assay with the allele-specific TaqMan probes allows to unambiguously determine the presence of

each of the 1401G>T alleles of *lhcgr* gene in the analyzed DNA sample and the animal's genotypes.

The real-time PCR method developed by us was tested on 195 samples of DNA from of Russian Black Pied cattle. The results of genotyping showed that 43,2% of cows carried the both alleles (GT genotype), 15,2% of cows were homozygous for T allele (TT genotype) and 41,6% – homozygous for G allele (GG genotype). Thus, in the studied population of cattle, the frequency of the G allele associated with higher rates in the total number of oocytes and the number of embryos survived after transplantation, as well as with the least number of unfertilized oocytes, was 63.2%.

Validation of the developed real-time PCR method was performed using PCR-RFLP analysis (Fig. 2). To identify the genotype of each animal, PCR products with 340-bp length were treated with endonuclease restriction NmuCI followed by electrophoretic analysis on an agarose gel (Fig. 2). The presence of the T allele leads to the emergence of the NmuCI endonuclease restriction site. The products of restriction analysis had the following lengths: for the GG genotype - 340 bp, for the TT genotype - 220/120 bp, for GT genotype - 340/220/120 bp. The results of the both methods are coincided, but method developed by us helps considerably shorten the time of analysis (up to 1 hour), which is an important advantage over PCR-RFLP analysis.

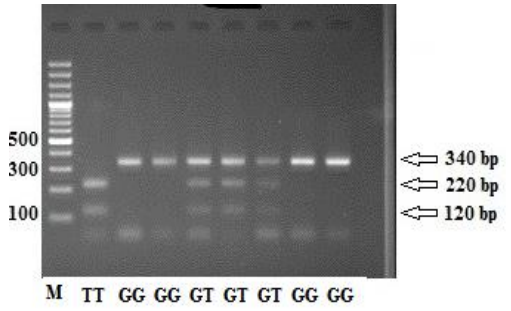

Fig. 2. The example of PCR-RFLP analysis of ss52050737 polymorphism of lhcgr gene in cattle.

IV. CONCLUSIONS

Thus, we developed an efficient and reliable method for rapid detection of ss52050737 polymorphism of *lhcgr* gene in cattle based on real-time PCR with allele-specific fluorescently labelled probes (TaqMan). This method allows genotyping up to 480 animals (depending on the model of a thermal cycler) for 1 hour and can be used in selection of donor cows with superovulatory potential.

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