DETECTION OF PROLACTIN RECEPTORS GENE PRLR-ALUI POLYMORPHISM, MARKER LITTER SIZE IN PIGS, USING HRM-ANALYSIS

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One of the most important indicators of the effectiveness of animal breeding is to increase the prolificacy of sows. For solving this problem is of considerable interest prolactin receptor gene (*PRLR*), which is used as a DNA marker pig fertility. At present for PRLR-AluI polymorphism detection using the PCR-RFLP method, which is quite expensive and time-consuming. In this study, for determining the PRLR-AluI polymorphism in the gene PRLR in pigs offered HRM-analysis technology. So the proposed method is based on the technology of HRM can be used as a rapid method for mass screening of animals.

Key words: technology HRM, PCR-RFLP polymorphism of *PRLR-AluI*, pig breeding

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DETECTION OF PROLACTIN RECEPTOR GENE POLYMORPHISM (PRLR-ALUI), PIG FERTILITY MARKER, USING HRM-ANALYSIS

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Summary. One of the most important efficiency parameter of animal breeding is an increase in multifetation of sows. For solving this problem prolactin receptor gene (*PRLR*), which is used as a DNA marker of pig fertility is of particular interest. At present the PCR-RFLP method, which is quite expensive and time-consuming, is used for PRLR-AluI polymorphism detection. The HRM-analysis technology was proposed in this study for detecting the PRLR-AluI polymorphism in the gene PRLR in pigs. So, the proposed method, based on the HRM technology, can be used as a rapid method for mass screening of animals for increasing multifetation of sows.

Key words: HRM technology, PCR-RFLP, *PRLR-AluI* polymorphism, alleles, pig breeding

Introduction. One of the most important efficiency parameter of animal breeding is an increase in the multifetation of sows. However, direct breeding for fertility is ineffective due to the low coefficient of inheritance (h = 0,1-0,3) [1]. Prolactin receptor gene (*PRLR*), which is located on chromosome 16 (SSC16), is considered as one of the pig fertility markers. Gene *PRLR* determines the specific receptor of anterior pituitary hormone – prolactin, which is involved in regulation of growth, metabolism and reproduction in mammals [2].

PRLR-AluI polymorphism was first discovered by Vincent AL in 1997.The relationship of this polymorphism with the number of piglets born alive in three genetic lines was also revealed [3], but the mechanism of the *PRLR* effect on the fertility of pigs remains unknown [4]. Currently, the traditional method of PCR-RFLP analysis, which is quite expensive and time-consuming, is used to detect

PRLR-AluI polymorphism . The aim of the research was to develop a reliable and inexpensive rapid method for detecting *PRLR-AluI* polymorphism using the HRM technology.

Materials and Methods. In this research, pigs of the following breeds were used as an object of study: Belarusian Large White, Belarusian Black-and-White, Yorkshire, Yorkshire factory type breed and Landrace. DNA isolated from whole blood served as a research material. The reagent kit "Nukleosorb" ("Primetech", Belarus) was used for DNA extraction. An animal sample n = (158) was analysed for *pPRLR-AluI* polymorphism of prolactin receptor gene. The amount of the isolated DNA was determined by Qubit® 2.0 Fluorometer. For a real time PCR and HRM-analysis, genomic DNA of samples was normalized (concentration of 4 ng / ml).

PCR-RFLP method. We have used the forward primer 5'-CGTGGCTCCGTTTGAAGAACC-3' and the reverse one 5'-CTGAAAGGAGTGCATAAAGCC-3' for amplification of the *PRLR* gene fragment of 163 bp in length (Drogemuller C., 2001) [5]. The reaction mixture of 20 µl contained deionized water, 1 × PCR buffer, 1.5 mM MgCl2, 200 µM of dNTP mixture, 300 nM of each primer, 1.5 U Taq-polymerase («Thermo scientific», Lithuania) and 50 ng of genomic DNA. Amplification was performed using the C1000TM Thermal Cycler («Bio-Rad», USA) under the following conditions: 94 ° C - 4 min; 30 cycles: 95 ° C - 30 sec, 58 ° C - 45 sec, 72 ° C - 40 sec; 72 ° C - 5 min. Restriction of the amplification products lasted for 6 hours at 37°C using AluI restrictase («Thermo scientific», Lithuania). Identification of the obtained fragments was performed in 2% agarose gel (SeaKem® LE) using the intercalating dye ZUBR Green-1 ("Primetech", Belarus).

DNA Sequencing. Specific PCR products of the studied PRLR gene locus were purified using the Silica Bead DNA Gel Extraction Kit («Thermo scientific», Lithuania) according to the attached manufacturer's instructions. The Big Dye Terminator v3.1 Cycle Sequencing Kit was used for conducting the sequencing PCR. The sequencing PCR was performed under the following conditions: 96° C -

1 min; 25 cycles: 96° C – 10 s, 50° C – 5 s, 60° C - 4 min; 16° C - 5 minutes. PCR products following PCR were purified by ethanol + Na_2EDTA reprecipitation. Sequencing was carried out using the 3500 Genetic Analyzer («Applied Biosystems», USA).

HRMA (High Resolution Melting Analysis). Samples of animal genomic DNA (n = 36) were analysed using HRM-assay techniques device CFX96 («BioRad», USA). The reaction mixture of the total 25 µl contained 1X PCR master mix ("Synthol", Russia) with the intercalating dye EvaGreen, 500 nM of each primer and 20 ng of DNA from each sample. Conditions for amplification and HRM-analysis were as follows: 95 ° C - 5 min; 35 cycles: 95 ° C - 10 sec, 58 ° C - 30 sec, 72 ° C - 15 sec; 94 ° C - 1 min, 72 ° C - 30 sec; Melt Curve 72 ° C - 95 ° C: Increment 0.2 ° C - 5 sec. Analysis of the results was made using the software for HRM analysis - Precision Melt Analysis ™ software.

Post-HRM sequencing. After identifying the genotypes by the HRM method 4 samples from each cluster with genotypes AA, BB, AB were sequenced. The PCR products after HRM-analysis were purified using Silica Bead DNA Gel Extraction Kit («Thermo scientific», Lithuania). Sequencing PCR was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit («Applied Biosystems», USA). Sequencing was carried out with the genetic analyzer 3500 («Applied Biosystems», USA).

Results and Discussion. Pig sample (n = 158) was investigated to determine the *PRLR-AluI* polymorphism by the PCR-RFLP method. Data on the occurrence frequencies of alleles and genotypes are shown in Table 1.

Table 1. Genetic structure of samples from populations of different pig breeds for *PRLR/AluI* gene

Genotypes	Landrace			Yorkshire			Large White Belarusian			Belarusian black-and-white			Belarusian type breed Yorkshire		
	F	T	X^2	F	T	X^2	F	T	X^2	F	T	X^2	F	T	X^2
AA	20	20,31	0,03	10	10,25	0,03	12	11,12	0,59	9	8,45	0,29	3	3,80	0,55
AB	25	24,38		20	19,49		10	11,77		8	9,10		11	9,40	

BB	7	7,31		9	9,26		4	3,11		3	2,45		5	5,80	
Allele	Allele frequencies ± Sp														
A	0,625±0,07			0,513±0,08			0,654±0,09			0,650±0,11			0,447±0,11		
B	0,375±0,07			0,487±0,08			0,346±0,09			0,350±0,11			0,553±0,11		

Note: F - the actual number of individuals, T - the theoretical number of individuals, X^2 - chi squared test.

As seen from Table 1, no significant differences were found in the allele distribution frequency for the gene *pPRLR / AluI* in the studied pig breeds that might be related to the peculiarities of their breeding. Only in pigs of Belarusian factory type Yorkshire the occurrence frequency of allele B was higher (0.553) than that of allele A, in contrast to the pig breed Yorkshire, Landrace, Belarusian black-and-white. All the populations studied were in a state of genetic equilibrium by the Hardy-Weinberg equilibrium. The results of PCR-RFLP analysis were obtained by the gel documentation Quantum ST4 system («Vilber lourman», France) and are presented in Figure 1.

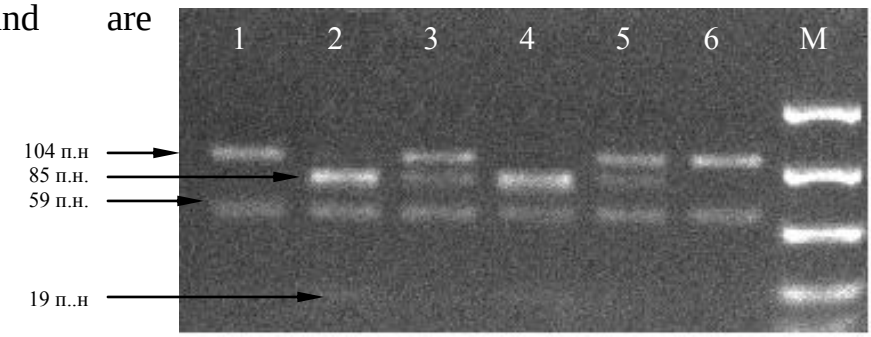

Fig.1. Detection of *pPRLR/AluI* polymorphism by PCR-RFLP method

Legend: M - marker FastRulerUltra Low Range DNA Ladder, lanes 2, 4 - homozygous genotype AA (85 bp, 59 bp 19 bp); lanes 3, 5 - heterozygous genotype AB (104 bp; 85 bp; 59 bp; 19 bp); lanes 1, 6 - homozygous genotype BB (104 bp; 59 bp).

Three samples with different genotypes (AA, AB and BB) were selected following PCR-RFLP analysis. These samples were sequenced for further use as the control ones for HRM-sample analysis. Data evaluation and processing were performed using the software “Sequencing Analysis, version 5.4” (Fig. 2).

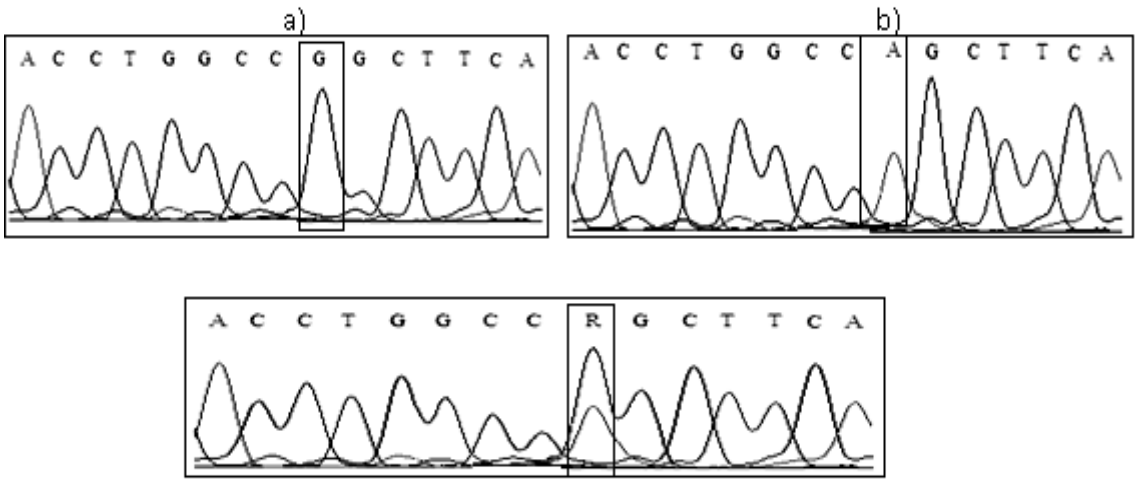

Fig. 2. Sample sequencing for detection of *pPRLR/AluI* polymorphism and determination of genotypes AA (a), IV (b) and AB (c)

The results of sequencing have revealed that *pPRLR/AluI* polymorphism was caused by the missense mutation G1789A (rs45435440), which results in an amino acid substitution Gly597Ser (GenBank DQ157757).

Out of the total sample of the investigated animals Yorkshire breed pigs were selected by the real-time PCR method using HRM-analysis. HRM analysis was performed using the software Precision Melt Analysis with melting of PCR product in a temperature range from 72 ° C to 95 ° C, where a change in the fluorescence intensity occurred whenever the temperature rises by 0,2 ° C for 5 sec.

To determine the genotypes of the *pPRLR/AluI* gene the melting curves, forming three distinct clusters corresponding to the genotypes AA, BB and AB, were used in the samples Division into clusters based on the HRM analysis results was confirmed by post-HRM sequencing, the data of which are shown on the right in Figure 3.

HRM-analysis is more efficient as compared with the PCR-RFLP method to detect *pPRLR/AluI* polymorphisms since it includes high sensitivity, low contamination risk, because the PCR and amplification product melting occur in one closed tube, the results are analyzed by the software excluding electrophoresis phase.

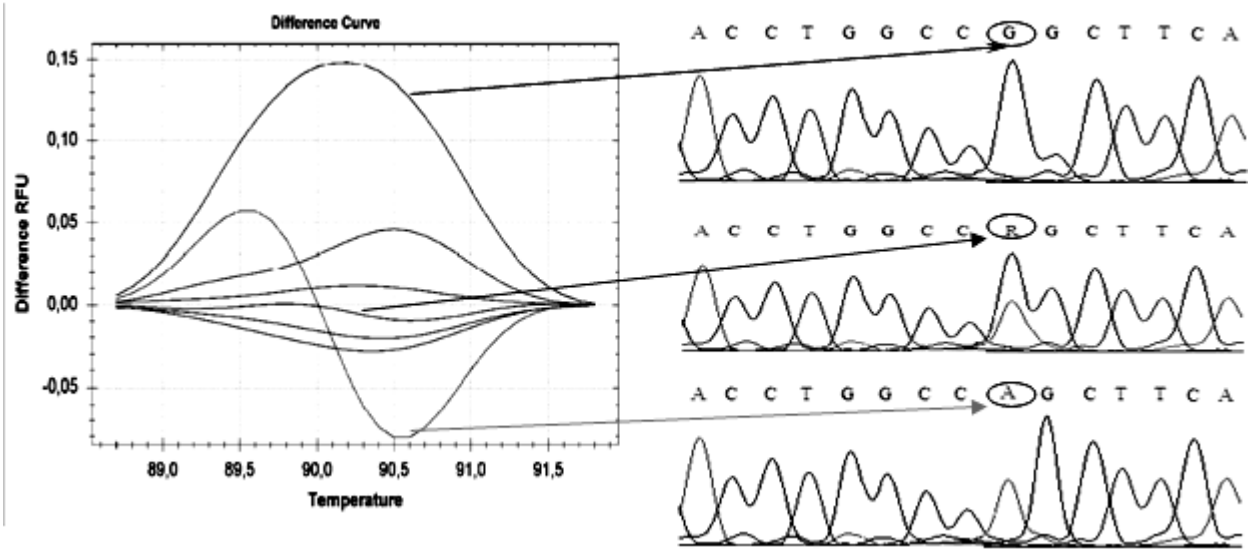

Fig. 3. HRM-analysis results in Precision Melt Analysis ™ software with the results of the post-HRM sequencing

Conclusion. HRM analysis technology is characterized by high sensitivity and specificity, is fast and inexpensive that allows its use as a rapid method to detect *pPRLR/ AluI* polymorphism for mass animal screening in selection and breeding work in pig breeding.

The development of molecular genetic studies and DNA technologies can identify preferable alleles and genotypes that makes it possible to predict the development of economically useful traits in animals for quick introduction of a desired genotype to the population of individuals to increase the profitability of pork production.

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