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Genotyping of Progesterone receptor gene in two rabbit breeds using PCR-RFLP assay

Milena Bozhilova-Sakova[1*], Denis Viryanski[1], Ivona Dimitrova[2] and Maya Ignatova[1]

[1] *Agricultural Academy, Institute of Animal Science, Department of Genetics, Breeding and Reproduction, 2232 Kostinbrod, Bulgaria*
[2] *University of Forestry, Agronomy Faculty, Department of Plant Protection, 1756 Sofia, Bulgaria*
[*]*Corresponding author:* bojilova_milena@abv.bg

Abstract

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The purpose of the present experimental work was to define the genetic status of the rabbit progesterone receptor gene (PGR), associated with reproduction traits and litter size in rabbits. For that matter were used 80 does of New Zealand White (NZW) and Californian (CA) rabbit breeds. Blood samples were collected in vacuum tubes and then stored at -20°C until DNA purification. The genetic polymorphism was determined by the PCR-RFLP method using the specific set of primers and the restriction enzyme Eco31I. In both breeds were identified all three possible genotypes (GG, AG and AA) of 2464G > A SNP for PGR gene, and in both studied breeds the frequency of homozygous genotype GG was higher than the other genotypes – 0.73 for NZW and 0.55 for CA. The genotype AA was with lowest frequency in both breeds – 0.12 for NZW and 0.07 for CA. The results in NZW breed were not consisted with HWE and there was observed statistically significant difference with $p = 0.005$. This is the first study in Bulgaria involving genetic diversity of PGR in rabbits. Further experiments are pending to associate the influence of each genotype with the litter size and milk production in rabbits.

Keywords: progesterone receptor gene; PCR-RFLP; polymorphism; rabbit breeding

Introduction

In the last 50 years, the production of rabbit meat in European countries has increased progressively, which puts Europe in second place after China in the production of rabbit meat in the world. In recent years of economic crisis, the industry has come under severe pressure, as rabbit meat is considered a delicacy rather than a staple food (Cullere & Zotte, 2018).

The rabbit is fast growing and early maturing animal, which is a significant advantage in raising animals in livestock farming. The meat has excellent dietary qualities including low fat content, which is valuable in the food industry of the developing countries. The short gestation period (pregnancy) of 30 days, early sexual maturation and the complete development of adult animals in around 6 months is ideal for the producers and consumers (Safaa et al., 2023).

The litter size is a crucial for rabbit breeding as they are raised mainly for meat production. Progesterone is a steroid hormone that is involved in reproductive processes, ovulation, implantation and pregnancy. The physiological role of progesterone is determined by its interaction with intracellular receptors, which exist in two isoforms – PR-A and PR-B (Ondruška et al., 2020).

Progesterone receptor gene (PGR) is located on the chromosome 1 from the rabbit genome. It consists of 10 exons in a region between 115,601,359 – 115,672,617 bp forward strands in the genome. The progesterone receptor gene encodes the progesterone receptor, which is found in different tissues of the uterus, mammary glands, brain, the

reproductive organs etc. (Source: UniProtKB/Swiss Prot; Acc:P06186).

Progesterone receptors are essential for uterine growth and receptivity to embryo implantation. Progesterone also acts in the release of mature oocytes, facilitation of implantation, and pregnancy. Different mutations in progesterone receptors could cause its dysfunction and could lead to implantation failure or early pregnancy loss in rabbits (Abd-Elkareem & Sayed Abou-Elhamd, 2019; Cope & Monsivais, 2022).

According to the authors after sequencing the gene, five SNPs were detected – one SNP in the PRG premotor region (g.2464 G > A), three SNPs in 5'-UTR of exon 1, and one SNP in exon 7. The first SNP (g.2464 G > A) produces two alleles (G and A) and three genotypes (GG, AG and AA). In several studies, the allele G was predominant, and the GG genotype had more implanted embryos, higher milk production, litter size, and number of weaned rabbits per litter than the other genotypes. The average number of stillborn kits per litter was significantly higher ($P < 0.05$) for AA compared to the GG genotype (Ramadan et al., 2020; Helal et al., 2024).

This study was conducted to establish the genetic diversity of progesterone receptor gene in does from two rabbit breeds (New Zealand white and Californian) raised in Institute of Animal Science – Kotinbrod.

Materials and Methods

Animals

Animals were raised under standard conditions of temperature, humidity, and photoperiod. The does were housed in enclosed one-floor building. Adult animals were housed separately in galvanized wire cages (40 × 60 × 50 cm^2), fitted with a nipple drinking system and a manual feeder. They were fed ad libitum with commercial pelleted feed and had free access to water. During the experimental work, all institutional and national guidelines for the care and use of animals were followed. All activities have been carried out in accordance with the recommendations contained in the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

DNA extraction

Genomic DNA was extracted from whole blood following the protocol Illustra Blood Genomic Prep DNA Purification Kit of GE Healthcare (UK), according to the manufacturer's instructions. The DNA concentration of each sample was measured by a Biodrop spectrophotometer. The quantity of the obtained DNA was about 10–20 ng/µl.

PCR amplification

DNA amplification was carried out by 96-Well thermal cycler (VerityPro, Applied Biosystem by Thermo Fisher Scientific) in a final volume of 20µL containing 8 µL of DNA template, 0.4 µL of ddH_2O, 0.8 µL of each primer (Fermentas) and 10 µL of 2 × (1.5 mM MgCl2) Red Taq DNA Polymerase Master mix (VWR, Int., Belgium). The primer set was suggested by Peiro et al. (2008) and was as followed:

PGR F: 5'-GAAGCAGGTCATGTCGATTGGAG-3'

PGR R: 5'-CGCCTCTGGTGCCAAGTCTC-3'

The PCR conditions were 94°C for 2 min, 94°C for 30 s, 66°C for 30 s, 72°C for 30 s, 35 cycles, with the last extension at 72°C for 10 min.

Restriction analysis

PCR products were digested by fast restriction enzyme Eco31I (Thermo Fisher Scientific, UK). The incubation was conducted in thermal block at 37°C for 15-20 min. The restriction fragments were separated by electrophoresis on 2.7% agarose gel stained by Red Gel Nucleic Acid Stain (Biotium) for 30-40 min under 120 V. They were visualized under ultraviolet transilluminatior and photographed in Hi-UVTM Duo Capture (HIMEDIA).

Statistical analysis

Allele and genotype frequencies were evaluated by the $\chi 2$-test and the Hardy-Weinberg equilibrium. The genetic diversity was calculated using the following parameters: expected heterozygosity (He), observed heterozygosity (Ho), effective allele number (AE) and coefficient of inbreeding (Fis).

Results and Discussion

As a result, in the present study could be reported that PCR-RFLP assay was suitable tool for genotyping and detection of genetic diversity in rabbit breeds raised in Bulgaria.

DNA samples were successfully extracted and purified from blood (Figure 1). After PCR amplification, fragments with an expected length of 558 bp were obtained (Figure 2). Restriction analysis revealed the presence of all three genotypes (GG, AG and AA) of 2464G > A SNP for PGR gene, and in both studied breeds the frequency of homozygous genotype GG was higher than the other genotypes – 0.73 for NZW and 0.55 for CA. The genotype AA was with lowest frequency in both breeds – 0.12 for NZW and 0.07 for CA.

Fig. 1. Extracted DNA samples with concentration approximately 10-12 ng/µl

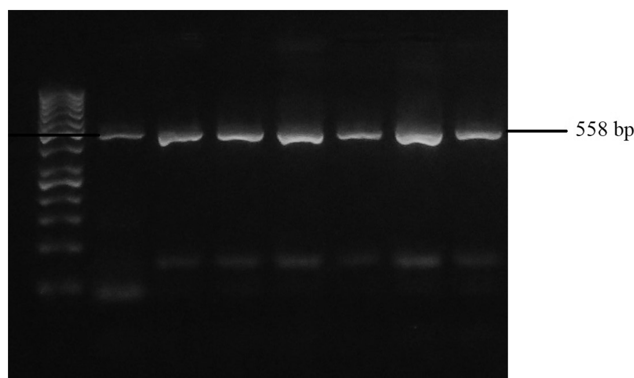

Fig. 2. PCR fragments of 2464G > A SNP of PGR gene

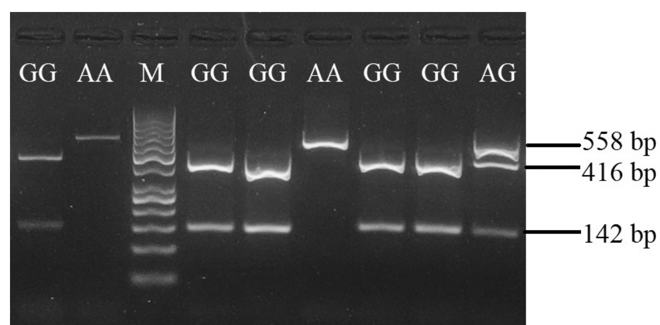

Fig. 3. Restriction fragments of PGR gene in animals from NZW and CA breed after digestion with Eco31I enzyme visualized on 2,7 % agarose gel

When comparing the observed and expected heterozygosity in NZW breed the population was not consisted with HWE and there was observed statistically significant difference with p = 0.005 (Table 1).

The effective number of alleles (A_E) was 1.60 for NZW and 1.89 for CA, where was closer to the cut-off value, which was typical of two-allele systems where both alleles effectively took a part.

The separated restriction fragments could be seen on Figure 3. The genotypes were determined based on the length of the different bands – genotype GG = 416 + 142 bp, AG = 558 + 416 + 142 bp and AA = 558 bp.

Similar to the present study, three different genotypes (AA, AG and GG) were detected in the progesterone receptor gene (PGR) promoter in a local Slovak crossbred rabbit line by Ondruška et al. (2020). The conducted study was to verify the effect of PGR gene polymorphism in relation to 21 days rabbit milk production, litter size, stillborn kits and pre-weaning mortality percentages at 35 days of age. 239 healthy adult animals (214 does and 25 bucks) of the local crossbred line of New Zealand White × Californian × Rabbit of Nitra were used. The highest reported frequency was registered for genotype AG (0.41), followed by genotype AA (0.33) and the lowest frequency was in genotype GG (0.26). The higher frequency of the allele A (0.53) vs allele G (0.47) was found by the analysis of the specific region of PRG receptor gene (558 bp). Their study noted that observed heterozygosity was 0.42 and the expected heterozygosity was 0.50. The scientific team reported that the GG genotype was found to be significant marker for the improving milk production and greater litter size.

Kotsyubenko et al. (2017) genotyped 60 animals of the California breed from High Litter Size (HLS) and Low Litter Size (LLS) groups. Using PCR-RFLP a 558-bp fragment of the rabbit progesterone receptor promoter region (PGR) gene was obtained. Differ to the present study they found that in individuals with high litter size dominated heterozygous genotype (0.70), while in animals with low litter size dominated genotype AA. The animals in the second group were characterized by very high observed heterozygosi-

Table 1. Breed, total number of animals, effective allele number, Allele frequency, genotype frequency, observed and expected heterozygosity, coefficient of inbreeding and chi-square

Breed	n	A_E	Allele frequency		Genotype frequency			Heterozygosity		Fis	X^2	p-value
			G	A	GG	AG	AA	Ho	He			
NZW	40	1.60	0.80	0.20	0.73	0.15	0.12	0.150	0.320	0.531	7.84	0.005*
CA	40	1.89	0.74	0.26	0.55	0.38	0.07	0.375	0.385	0.240	0.00	1.00

*Statistically significant difference

ty (Ho = 0.700), which significantly exceeds the expected heterozygosity (He = 0.455). Differ to the present study the authors reported for the presence of two genotype – heterozygous AG and homozygous AA.

The Egyptian Synthetic Rabbit Line called the Moshtohor (M-line) line and their parents from the Spanish V-line and the Sinai Gabali rabbit breed were also studied by PCR-RFLP. Restriction analysis was conducted with endonuclease Eco31I and three genotypes were revealed – GG, AG and AA. The genotype frequency of AG ranged from 0.68 in the V-line to 0.88 in FGP and in contrast to the present study, it was higher than the genotypes GG and AA and in all studied populations. The frequency of the A allele was higher than the G allele in all populations, except for the M-line, where the G allele had the highest allele frequency of 0.54, as announced in the following experiment. The highest effective number of alleles (Ne) for SNP of the PGR gene was recorded for the M-line (1.987), followed by FGP (1.972), while the lowest number was recorded for the V-line (1.891). The four populations studied were not in Hardy-Weinberg equilibrium ($P < 0.05$ or $P < 0.001$). The average observed (Ho), expected (He) heterozygosity and polymorphic information content (PIC) were 1.943, 0.485 and 0.367, respectively (El-Aksher et al., 2016).

Conclusions

This was a pioneer investigation related to the genetic diversity of the progesterone receptor gene involving rabbit breeds raised in Bulgaria. As a result, it could be announced that in the studied groups of New Zealand White and Californian rabbits were observed all three possible genotypes – GG, AG and AA. The frequency of homozygous wild genotype GG was higher than the other genotypes – 0.73 for NZW and 0.55 for CA. The mutant genotype AA was with lowest frequency in both breeds – 0.12 for NZW and 0.07 for CA. The results in NZW breed were not consisted with HWE and there was observed statistically significant difference with $p = 0.005$. Further experiments are pending to associate the influence of each genotype with the litter size and milk production in rabbits.

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