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PCR DETECTION METHOD AND SANGER SEQUENCING FOR PRNP GOAT SUSCEPTIBILITY

METODA PCR DE DETECȚIE ȘI SECVENȚIEREA SANGER A SUSCEPTIBILITĂȚII GENEI PRNP LA CAPRINE

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ABSTRACT | REZUMAT

The genetic susceptibility of goats to scrapie has been less investigated and analysed to determine the polymorphism of the PRNP gene, a polymorphism that is associated with resistance or susceptibility to scrapie in sheep. In goats, studies in the field of scrapie susceptibility have been carried out by countries with a tradition in the exploitation of this species, such as Greece, France, Italy, and Great Britain, or where the incidence of scrapie in goats is high (e.g., Cyprus). These studies have shown that allelic variation of the PRNP gene can modulate susceptibility to scrapie in goats. The present paper aims to optimize the classical method of PCR detection of the PR NP gene in goats, and SANGER sequencing of the PRNP gene to highlight the alleles of interest in three goats breeds in Romania: Carpathian breed, Banat White, and French Alpine breed. PureLink Genomic DNA Kit was used for DNA extraction from blood samples and hair follicles. The amplification kit used was Amplitaq Gold 360-DNA Polymerase-250U kit (Invitrogen, Thermo Fisher Scientific, Applied Biosystems,US).The sequencing reactions were done by outsourcing to a specialized company that performs Sanger Sequencing (Antisel, CEMIA). Big Dye Terminator Cycle Sequencing Kit v3.1 and ABI PRISM 3130 (Applied Biosystems) were used for sequencing. 156 blood samples and 156 hair samples were tested by classic PCR, 50 Carpathian breed goats, 53 French Alpine breed goats, and 53 Banat White breed goats. For the blood samples, the amplification bands were very intense, which correlates with the amount of DNA. All blood and hair samples tested showed amplification bands at 1200 bp according to primers. The sequences were free of impurities which proves that the genetic material obtained from the hair with root even if it was in a smaller DNA amount was enough to obtain the PRNP gene sequence. Following these determinations, different alleles of the PRNP gene and different genotypes were obtained, which demonstrate both the high genetic sensitivity and genetic resistance to scrapie.

Keywords: goats**,** PRNP susceptibility, PCR

Susceptibilitatea genetică a caprelor la scrapie a fost mai puțin investigată și analizată pentru a determina polimorfismul genei PRNP, un polimorfism care s-a dovedit a fi asociat cu rezistența sau susceptibilitatea la scrapie la ovine. La capre, studii în domeniul susceptibilității scrapiei au fost efectuate de țări cu tradiție în exploatarea acestei specii, precum Grecia, Franța, Italia și Marea Britanie, sau unde incidența scrapiei la capre este mare (ex. Cipru). Aceste studii au arătat că variația alelică a genei PRNP poate modula susceptibilitatea la scrapie a caprelor. Lucrarea de față își propune să optimizeze metoda clasică de detecție prin PCR a genei PRNP la capre și secvențierea SANGER a genei PRNP pentru a evidenția alelele de interes la trei rase de capre din România: rasa Carpatina, rasa Alba de Banat și rasa Alpină Franceză. PureLink Genomic DNA Kit a fost folosit pentru extractia ADN din probe de sânge și foliculi de păr cu rădăcină. Kitul Amplitaq Gold 360-DNA Polymerase-250U kit (Invitrogen, Thermo Fisher Scientific, Applied Biosystems, US) a fost folosit pentru amplificarea prin PCR clasic.Reacțiile de secvențiere au fost realizate prin externalizare către o firmă specializată care realizează secvențierea Sanger (Antisel, CEMIA). Kitul de secvențiere Big Dye Terminator Cycle v3.1 și ABI PRISM 3130 (Applied Biosystems) au fost folosite pentru secvențiere. Aici au fost testate 156 de probe de sânge și 156 de probe de păr prin PCR clasic: 50 probe de la capre de rasă Carpatină, 53 de probe de la capre de rasă Alpină Franceză și 53 de probe de la capre de rasă Alba de Banat. Pentru probele de sânge, benzile de amplificare au fost foarte intense, ceea ce se corelează cu cantitatea de ADN. Toate probele de sânge și de păr testate au prezentat benzi de amplificare la 1200 bp conform primerilor. Rezultatele obținute au fost adecvate, secvențele au fost lipsite de impurități ceea ce demonstrează că materialul genetic obținut din părul cu rădăcină, chiar dacă a fost într-o cantitate mai mică de ADN, a fost suficient pentru a obține secvența genei PRNP. În urma acestor determinări s-au obținut diferite alele ale genei PRNP și diferite genotipuri, care demonstrează atât sensibilitatea, cât și rezistența genetică a caprelor cercetate la scrapie.

Cuvinte cheie: Capre, susceptibilitatea PRNP, PCR

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The genetic susceptibility of goats to scrapie has been less investigated and analysed to determine the polymorphism of the PRNP gene, a polymorphism that has been shown to be associated with resistance or susceptibility to disease in sheep. In sheep, PRNP gene polymorphism at codons 136 (A or V), 154 (R or H), and 171 (R, Q, or H) decisively modulate disease susceptibility (10).

In goats, studies in the field of scrapie susceptibility have been carried out by countries with a tradition in the exploitation of this species, such as Greece, France, Italy, and Great Britain, or where the incidence of scrapie in goats is high (e.g., Cyprus). These studies have shown that allelic variation of the PRNP gene can modulate susceptibility to scrapie in goats (1, 10). In goats, strong polymorphisms have been described, such as V21A, L23P, G49S, W102G, T110P, G127S, I142M, H143R, N146S, P168Q, R211Q, Q220H, Q222K, P240S (1, 10, 15) and many others, without being clearly and completely defined susceptible / resistant genotypes.

There are approximately 30 polymorphisms in the PRNP gene, but only codons I142M, H143R, N146S/D, H154R, R211Q, and Q222K were associated with susceptibility in goat scrapie (1, 15). According to recent studies, alleles 222K, 146S, and 211Q are associated with the highest degree of scrapie resistance and heterozygosity at codons I142M, H143R, N146S, R211Q, and Q222K proved to confer decreased susceptibility to scrapie (1, 2, 8, 15). Specimens with this allele are the best candidates for selective breeding programs in goats and this issue is also supported by the European Food Safety Authority (13).

In Romania there are no regulations for selective breeding programs for goats for scrapie control and this is correlated with the lack of information and investigations to know the PRNP polymorphisms in goats from Romania.

In other countries such as China, Japan, Italy, France, the United Kingdom, Cyprus, and Turkey, information has already been reported on the genotypes of the PRNP gene associated with scrapie in goats (9). Based on this information, EFSA has proposed national genetic selection programs for goats for breeds of interest (13). Analysing the diversity of goat breeds resident in Romania, we find the existence mainly of the Carpathian breeds, a native breed that comes from the Prisca breed, and Banat White. There are also many farms with imported breeds, such as the French Alpine, Angora, and Saanen.

The present paper aims to optimize the classical method of PCR detection of the PRNP gene in goats, and Sanger sequencing of the PRNP gene in order to highlight the alleles of interest in three goats breeds from Romania: Carpathian, Banat White, and French Alpine.

MATERIALS AND METHODS

Animals and biological samples

Whole blood samples and hair follicles were collected from 50 Carpathian breed goats from Dudescu village (Braila County, Romania), 53 French Alpine breed goats from Gara Banca (Vaslui County, Romania), and 53 Banat White breed goats from Ion Neculce (Iasi County, Romania).

Blood samples were collected in 5 ml K3-EDTA tubes. Hair samples were collected in sterile 50 ml tubes in a number of 30-40 strands of hair with follicles per sample. Blood samples were stored at -20°C until DNA extraction and hair samples were stored at room temperature.

DNA extraction from blood and hair follicles

PureLink Genomic DNA Kit was used for DNA extraction from blood samples and hair follicles (5). The protocol used for blood DNA extraction and hair follicles was previously described by Gurau et al. (2021) (4). The samples were stored at -20°C until the quantifcation step and amplification step.

DNA quantification

Qubit dsDNA HS test kit (Invitrogen, Thermo Fisher Scientific, Applied Biosystems, US) was used to quantify the DNA extract from blood and hair follicles. Quantification of DNA was made by Qubit 4 fluorometer (6). The protocol used for DNA quantification from blood and hair follicles was previously described by Gurau et al. (2021) (4).

DNA amplification of PRNP gene

The amplification kit used was Amplitaq Gold 360-DNA Polymerase-250U kit (Invitrogen, Thermo Fisher Scientific, Applied Biosystems,US). The reagents were 5 µl of extracted DNA, 5 µl of 25 mM MgCl2, 5 µl of 360 GC Enhancer, 1 µl of 40 µM dNTPs, 0.5 µl of 25 pmol/µl primers, 5 µl of 10X AmpliTaqGold® PCR Buffer and 2.5 units of AmpliTaq Gold® (Applied Biosystems). The primers (Table 1) used were previously described by Vaccari et al. (2009) and Migliore et al. (2015) (11, 17). The temperature protocol used was as follows:5 min at 96 °C, 30 sec at 96 °C, 15 sec at 57 °C, 90 sec at 72 °C for 40 cycles, and a final cycle of 4 min at 72 °C.

The PCR product was subjected to agarose gel electrophoresis to visualize DNA amplicons before Sanger sequencing. Agarose gel was 1.5% agarose in TAE / TBE, 0.9 g agarose with 60 ml TAE / TBE 1x and 5 µl EtBr 10 mg/ml. It was charged 2 µl loading buffer with 10 µl amplicons. Migration was made at 90V; 1.5A; 35 min. Also, 10 µl of 10 mg EtBr/ml was added to the migration buffer. The fragment migrated at ~ 1200 bp.

Table 1

Primer's sequences used for classical PCR of goat PRNP gene (11, 17)

Primer name	Sequence	Genome position / bp
F1	5`- CATTTATGACCTAGAATGTTTATAGCTGATGCCA - 3`	PRNP CDS ORF 1200 bp
	5`- TTGAATGAATATTATGTGGCCTCCTTCCAGAC -3`	PRNP CDS ORF 1200 bp

Table 2

Primer's sequences used for Sanger sequencing of goat PRNP gene (11, 17)

Primer name	Sequence	Genome position / bp
T3	5 '-TTTACGTGGGCATTTGATGC-3'	ORF
T4	5 '-GGCTGCAGGTAGACACTCTC-3'	ORF

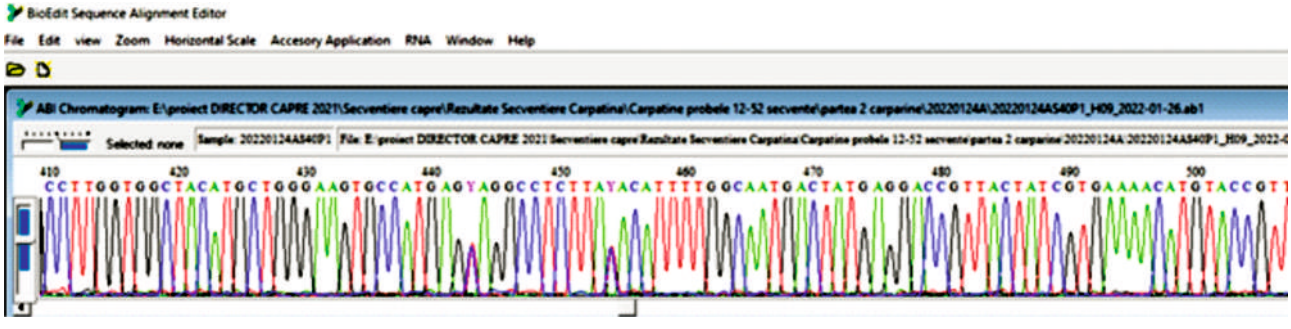

Fig. 1. BioEdit Software used for processing of the Sanger sequencing samples

DNA Sanger sequencing of PRNP gene

The sequencing reactions were done by outsourcing to a specialized company that performs Sanger sequencing (Antisel, CEMIA). Big Dye Terminator Cycle Sequencing Kit v3.1 and ABI PRISM 3130 (Applied Biosystems) were used for sequencing.

The primers used for Sanger sequencing (Table 2) were previously described by Vaccari et al. (2009) and Migliore et al. (2015) (11, 17).

Alignment of the sequence and its processing after the sequencing step was performed with BioEdit Software (Fig. 1).

RESULTS AND DISCUSSIONS

All 156 blood samples and 156 hair samples from which DNA was extracted were quantified with a Qubit fluorometer. The amounts of DNA isolated from blood were between 6.8 ng/ µl and 32.4 ng/ µl. The amounts of DNA isolated from hair follicles were between 1.09 ng/µl and 29.9 ng/µl (Table 3). The quantities of DNA in the hair follicles were less than in the blood samples. This is also supported in the next stage of amplification by classical PCR where the amplification bands are low intensity in the case of root hair samples. However, the samples with very small amounts of DNA had amplification bands, which proves that the hair follicles samples are suitable for PCR detection. In other scientific papers, there were obtained DNA quantities between 49 ng/µL and 72 ng/µL from hair follicles (3) and 50 ng/µL and 150 ng/µL (12), which shows that our results were approximatively in the same values.

So, all 156 blood samples and 156 hair follicle samples were tested by classic PCR. Both blood samples and root hair samples were taken from the same animal, from which DNA was subsequently extracted. After the extraction, the classical PCR reaction was performed from blood and hair samples. All 156 blood samples and 156 hair follicle samples tested showed amplification bands at 1200 bp according to primers. For the blood samples, the amplification bands were very intense, which correlates with the amount of DNA extracted and quantified with the Qubit fluorometer (Fig. 2). For the root hair samples, the amplification bands were of different intensities, some samples having a low intensity which is in agreement with the amount of DNA extracted from the sample (Fig. 3). For each batch of amplified samples, a negative reaction control was used, with the role of validating the reaction. In our study all negative controls haven't showed amplification bands, all the reactions were validated.

After classical PCR amplification of the samples, Sanger sequencing was performed to obtain the DNA sequence associated with the PRNP gene. The sequencing was done by CEMIA company and the results were processed with BioEdit Software. Appropriate sequences were obtained, without errors and without the presence of impurities, which shows that the purification of the samples was done correctly. All sequences obtained could be processed on both the for-

Table 3
DNA quantification from blood and hair follicles

No.	Sample name	Sex	Whole blood ng/µL	Whole blood ng/ml	Hair follicle ng/µl	Hair follicle ng/ml
1	1C	M	28.4	426	3.77	56.5
2	2C	M	13.2	198	9.27	139
3	3C	M	7.67	115	12.1	182
4	4C	M	15.3	229	21.7	326
5	5C	M	16.3	245	18.3	274
6	6C	M	17.3	260	6.37	95.5
7	7C	M	6.8	102	24.8	372
8	8C	M	15.4	231	25.9	388
9	9C	M	18.5	278	29.9	449
10	26C	M	30.1	451	15.6	234
11	27C	M	3.21	48.2	18.6	279
12	28C	M	4.53	67.9	18.4	276
13	29C	M	22.9	343	20.7	311
14	30C	M	32.4	486	1.09	15.9
15	10C	F	24.4	366	3.73	55.9
16	11C	F	17.1	256	34.7	520
17	12C	F	25.1	376	28.3	424
18	13C	F	8.6	129	3.04	45.6
19	14C	F	14	210	8.8	132
20	15C	F	6.8	102	6.65	99.7
21	16C	F	13.1	197	9.13	137

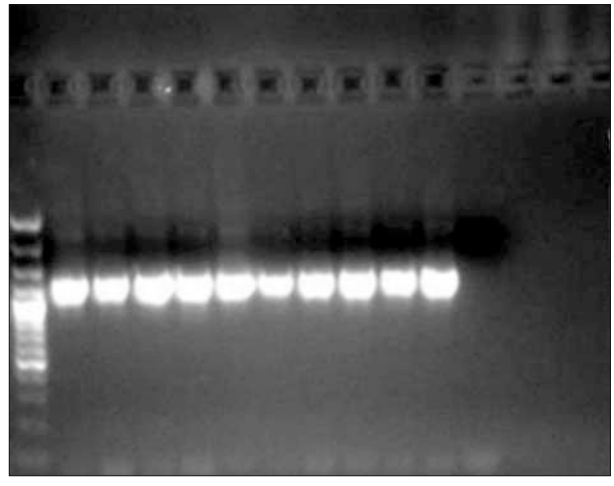

Fig. 2. Electrophoresis UV image of blood samples PCR amplification

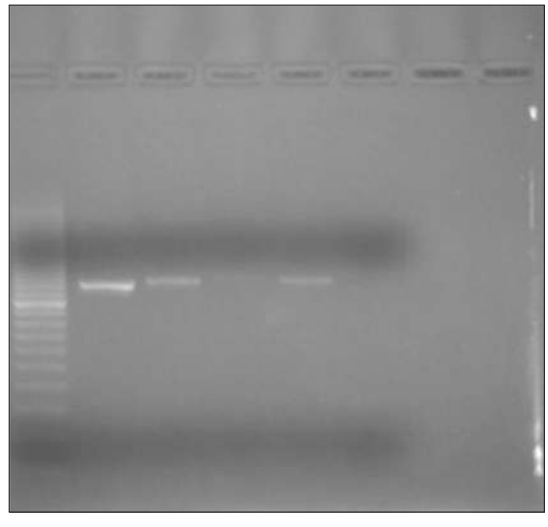

Fig. 3. Electrophoresis UV image of hair follicle samples PCR amplification

ward sequence and the reverse sequence. With the help of the BioEdit Software, it was possible to process complete sequences of nucleotides which were then transformed into amino acids so that the codons of interest could be observed. After obtaining the amino acid sequence, the codons that were studied were: 37, 102, 110, 110, 127, 142, 142, 143, 146, 151, 154, 168, 173, 173, 211, 218, 222, and 240.

The Sanger sequencing method is a gold standard method for determining the interest codons of the PRNP gene in goats. (7, 14, 16).

The results obtained by the sequencing method from the hair follicles samples were adequate, the sequences were free of impurities which proves that the genetic material obtained from the hair with root even if it was in a smaller DNA amount was enough to obtain the PRNP gene sequence. Following these determinations, different alleles of the PRNP gene and different genotypes were obtained, which demonstrate both the high genetic sensitivity to goat scrapie and their genetic resistance to scrapie.

CONCLUSIONS

The method of extracting DNA from hair follicles is suitable for genetic determinations in a large number of animals being a non-invasive method. Detection of the PRNP gene in goats by the Sanger sequencing is a gold standard method. The DNA amount obtained from the hair follicles with root even if it was in a smaller DNA amount was enough to obtain a good and processable PRNP gene sequence with the BioEdit Software.

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