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Original Scientific Article

POLYMORPHISM OF PRION PROTEIN GENE AND EVALUATION ON GENETIC RISK FOR SCRAPIE IN THREE NATIVE AND ONE INTRODUCED BREED IN THE REPUBLIC OF NORTH MACEDONIA

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ABSTRACT

Transmissible spongiform encephalopathy in sheep is known as scrapie. Polymorphisms at codons 136, 154, and 171 of the prion protein (PRNP) gene are associated with resistance or susceptibility to the disease. Based on allele combinations, sheep are classified into five genetic risk groups. The aim of this study was to determine the distribution of PRNP gene polymorphisms and assess the genetic susceptibility to scrapie among native and introduced sheep breeds reared in North Macedonia. A total of 309 unrelated animals from three native Pramenka-type sheep breeds, Karakachanian (n=92), Ovchepolian (n=91), and Sharplaninian (n=34), and one introduced breed, Awassi (n=92), were genotyped. The Awassi and Sharplaninian breeds were dominated by the ARQ/ARQ genotype (R3). In Karakachanian sheep, the ARR/ARQ genotype (R2) was most frequent, while Ovchepolian sheep showed equal predominance of ARR/ARQ (R2) and ARQ/ARQ (R3). High-risk genotypes (R4 and R5) were detected only in the Ovchepolian and Sharplaninian breeds. The ARQ allele was the most frequent in Awassi (0.7609), Ovchepolian (0.4945), and Sharplaninian (0.5000), whereas the ARR allele predominated in Karakachanian sheep (0.5772). The VRQ allele was detected only in the Ovchepolian and Sharplaninian breeds. These findings demonstrate genetic differences among the studied sheep populations and provide valuable information for breeding strategies aimed at increasing resistance to scrapie while conserving breed diversity.

Keywords: scrapie, polymorphism, PrP genotype, Pramenka, Awassi

INTRODUCTION

Scrapie is a fatal neurodegenerative disease affecting sheep and goats and belongs to a group of diseases known as transmissible spongiform encephalopathies (TSEs). Diseases within this group are also referred to as prion diseases, in which the neuronal surface glycoprotein known as the prion protein (PrP) is converted into its abnormal, protease-resistant isoform (PrP^Sc). This abnormal isoform becomes the infectious agent and accumulates in the central nervous system and lymphoid tissues (1). The sheep prion protein is the product of the PrP gene and consists of 250 amino acids. Numerous studies have demonstrated that polymorphisms in the PrP gene are closely associated with genetic susceptibility to scrapie in sheep. More than 32 codons in the sheep PrP gene have been identified as polymorphic (2), however, polymorphisms at codons 136, 154, and 171 are strongly associated with the degree of resistance or susceptibility of the sheep genome to classical (typical) scrapie (3). Codon 171 was the first identified as polymorphic codon in the sheep genome in relation to scrapie. At this position, arginine (R), histidine (H), or glutamine (Q) may occur (2, 4, 5). Polymorphisms at two additional codons (A136V and R154H) have also been shown to be strongly associated with resistance to classical scrapie. At codon 136, alanine (A) is associated with resistance, whereas valine (V) is associated with susceptibility (6). Results from numerous studies (7, 8, 9, 10) highlight the importance of polymorphism at codon 154 of the sheep PrP gene.

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Different authors have interpreted the impact of this polymorphism differently. Some report that the presence of histidine (H) at codon 154 is associated with increased susceptibility to disease, while the presence of arginine (R) is associated with increased resistance (10). In contrast, other authors (8, 9, 10) argue that arginine (R) at codon 154 alone has no major impact on disease susceptibility or resistance, but that its interaction with codon 171 influences the natural occurrence of scrapie. This same group suggests that when codon 154 encodes histidine (H), it leads to increased resistance to disease, thereby reducing the importance of codon 171 when it encodes glutamine (Q) (11). The most widely accepted explanation for the role of codon 154 considers its interaction with codon 171. Furthermore, clustering of the codon 154 based on the encoded amino acid provides additional insight into its influence (9). If codon 154 encodes histidine (H) and codon 171 encodes arginine (R), homozygous animals belong to the most vulnerable group. Heterozygous animals (H154R171/x154x171) belong to a less sensitive group, while animals lacking the H154R171 combination belong to the most resistant group (9). Polymorphisms at these three codons result in five different alleles (ARQ, VRQ, AHQ, ARR, and ARH), leading to 15 different genotypes associated with resistance or susceptibility to the classical form of the disease (2). Sheep with the ARR genotype are resistant to scrapie, whereas animals carrying the VRQ allele are highly susceptible and exhibit a short survival period (2, 4). The application of the National Scrapie Plan (NSP) in the United Kingdom provided a clear classification of the 15 sheep genotypes according to their susceptibility to classical scrapie (12). According to this classification, genotypes are grouped into five risk categories (R1, R2, R3, R4, and R5) (3).

The main aim of the European Community regulation for scrapie breeding programs is to increase the frequency of the resistant ARR allele and decrease the frequency of the VRQ allele, which is highly susceptible to the classical form of scrapie (13). In order to protect breed integrity and production traits, derogations from the measures outlined in Decision 2002/1003/EC may be applied. Specifically, derogations are allowed for breeds that display a low frequency or absence of the ARR allele (below 25% in surveys, or an absence or level below 10% in the genome), as well as for breeds that are endangered or at risk of being lost to farming (13).

This study represents the first attempt to characterize PrP gene polymorphisms related to classical scrapie in three native sheep breeds and one introduced breed in the Republic of North Macedonia. Furthermore, based on genotype and allele frequencies, the secondary aim was to assess genetic resistance or risk to scrapie and to propose future breeding strategies for the analyzed breeds. To date, there have been no officially reported cases of scrapie in the Republic of North Macedonia, therefore, case–control studies and genotype-specific disease incidence could not be performed.

MATERIAL AND METHODS

Material

A total of 309 unrelated animals from three native Pramenka-type sheep breeds, Karakachanian (n=92), Ovchepolian (n=91), and Sharplaninian (n=34), and one introduced breed, Awassi (n=92), were included in this study. All animals were reared on privately owned farms. The Awassi breed is well known and has been thoroughly described (14). The uniqueness of the genome of native sheep populations in Republic of North Macedonia is clearly differentiated at phenotypic (15) and molecular level (16). Blood samples were collected from the jugular vein (*v. jugularis*) into EDTA-containing vacutainer tubes, transported in an icebox, and stored at -20 °C until further processing.

Ethical statement

The study was carried out following the principles and ethical approaches of FAO guideline for molecular genetic characterization of animal genetic resources (17). All procedures involving animals were conducted in accordance with the applicable national legislation and internationally accepted guidelines governing the welfare, care, and use of animals for scientific research. Blood samples were collected by the authorized State Veterinary Service during routine annual health monitoring of the animals. Sampling was performed by qualified personnel in a manner that minimized animal stress and discomfort. The farmers/breeders participating in this study were informed of the obtained results and provided their consent for the publication of the results.

Methods

DNA extraction

Genomic DNA was extracted using the phenol–chloroform extraction method followed by ethanol precipitation (18).

Real-time PCR

Unlabeled PCR primers (forward 5′-AGG CTG GGG TCA AGG TGG TAG C-3′ and reverse 5′-TGG TAC TGG GTG ATG CAC ATT TGC-3′) and TaqMan® minor groove binder (MGB) probes labeled with FAM™ and VIC® dyes were used for polymorphisms at codon 136, 154 and codon 171 (19). Unlabeled PCR primers and TaqMan® MGB probes were designed for each of the analyzed polymorphisms. In each PCR reaction, two allelic variants of a given codon were determined simultaneously using two allele-specific probes, each labeled with a different fluorophore. Four separate PCR reactions were prepared for each DNA sample, targeting polymorphisms at codon 136, codon 154, codon 171 (R/Q), and codon 171 (H/Q). All PCR reactions were performed on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) using TaqMan® Universal PCR Master Mix, primers, allele-specific probes, and genomic DNA. The PCR protocol consisted of an initial TaqMan® polymerase activation and denaturation step (10 min at 95 °C), followed by 40 amplification cycles of denaturation (15 sec at 95 °C) and annealing/extension (1 min at 60 °C). Fluorescent signals generated by cleavage of the MGB probes were detected in real time during the annealing/extension phase. Data analysis was performed using ABI PRISM® 7000 SDS software version 1.1, applying component analysis.

DNA sequencing

Sequencing analysis was conducted to confirm the detected polymorphisms at the targeted codons. PCR products were purified using ExoSAP-IT™ (GE Healthcare Bio-Sciences Ltd.). Purified amplicons were sequenced using BigDye® Terminator chemistry on an ABI 3100 Avant Automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were analyzed with Sequencing Analysis Software version 3.3 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis of the data was performed using the software package CERVUS version 3.0.3 (20).

RESULTS

Allele and genotype frequencies

Analysis of PrP polymorphisms at codons 136, 154, and 171 revealed different distributions of ARR, ARQ, ARH, AHQ, and VRQ alleles in the genomes of the analyzed sheep populations. Three alleles were detected in the Awassi (ARR, ARQ, ARH), four alleles in the Karakachanian pramenka (ARR, ARQ, ARH, AHQ), whereas all five alleles were present in the Ovchepolian and Sharplaninian pramenka (ARR, ARQ, ARH, AHQ, and VRQ) (Table 1).

The ARQ allele was the most frequent in the Awassi breed, Sharplaninian and Ovchepolian pramenka (0.7609, 0.5000 and 0.4945, respectively). In contrast, the ARR allele predominated in the Karakachanian pramnenka (0.5762). The most susceptible allele, VRQ, was detected only in the genome of Ovchepolian and Sharplaninian pramenka (Table 1).

Table 1. Allele frequencies in analyzed populations

Allele	Awassi (n=92)	Karakachanian (n=92)	Ovchepolian (n=91)	Sharpalninian (n=34)
	Allele frequencies			
ARR	0.0054	0.5762	0.3407	0.3383
ARQ	0.7609	0.4130	0.4945	0.5000
ARH	0.2337	0.0054	0.0110	0.0735
AHQ		0.0054	0.0824	0.0588
VRQ			0.0714	0.0294

Eleven genotypes were observed across the studied populations, comprising combinations of the five alleles (Table 2). The ARQ/ARQ genotype was present in all breeds, and was the predominant genotype in the Awassi population (0.5870). In the Sharplaninian pramenka, two genotypes, ARQ/ARQ and ARR/ARQ, were equally prevalent (0.2647 each). The ARR/ARQ genotype was predominant in the Karakachanian and Ovchepolian pramenka, with frequencies of 0.5435 and 0.2857, respectively (Table 2).

The most resistant genotype, ARR/ARR, was present only in the genome of the native Pramenka-type breeds and was completely absent in the Awassi breed (Table 2). Genotypes ARR/VRQ and VRQ/ARQ were observed only in the Ovchepolian and Sharplaninian pramenka. The ARH/ARH genotype was exclusive to the Awassi breed (Table 2).

In the Awassi breed, the lowest-frequency genotype was ARR/ARQ, whereas in Karakachanian pramenka, the lowest frequencies were observed for AHQ/ARQ and ARH/ARQ genotypes. In the Ovchepolian pramenka, three genotypes (ARR/ARH, AHQ/AHQ, and ARH/ARQ) were equally rare, with ARR/ARH and AHQ/AHQ present exclusively in this population. In the Sharplaninian pramenka, for the following three genotypes AHQ/ARQ, ARR/VRQ, and VRQ/ARQ lowest frequencies were observed (Table 2).

Table 2. Genotype frequencies in analyzed populations

Genotype	Awassi	Karakachanian	Ovchepolian	Sharpalninian	Level of resistance
	Genotype frequency				
ARR/ARR		0.3043	0.1320	0.1470	Highly resistant
ARR/AHQ			0.0659	0.0882	Resistant
ARR/ARH			0.0110		Resistant
ARR/ARQ	0.0109	0.5435	0.2857	0.2647	Resistant
AHQ/AHQ			0.0110		Little resistant
AHQ/ARQ		0.0109	0.0879	0.0294	Little resistant
ARH/ARH	0.0651				Little resistant
ARH/ARQ	0.3370	0.0109	0.0110	0.1472	Little resistant
ARQ/ARQ	0.5870	0.1304	0.2527	0.2647	Little resistant
ARR/VRQ			0.0549	0.0294	Susceptible
VRQ/ARQ			0.0879	0.0294	Highly susceptible

Distribution of scrapie risk groups

Data regarding the distribution of scrapie risk groups (R1-R5) across the four populations indicate clear differences in genetic susceptibility and dominance of specific risk groups in each population (Table 3). In the Awassi, the R3 risk group was dominant (98.91%), indicating a homogeneous presence of medium-risk genotypes, while the R2 group was minimally represented (1.09%). Other risk groups were absent, reflecting limited genetic variability for scrapie susceptibility. In the Karakachanian pramenka, the R2 group was most frequent (54.35%), followed by R1 (30.43%) and R3 (15.2%). This distribution suggests greater heterogeneity, with both low and medium-risk genotypes present (Table 3). The Ovchepolian pramenka displayed a more balanced distribution between R2 and R3 (36.3% each), with smaller contributions from R1 (13.19%), R4 (5.49%), and R5 (8.8%). The presence of R4 and R5 genotypes indicates higher-risk animals, which may be relevant for selection and control strategies. In the Sharplaninian pramenka, R3 was the most common group (44.12%), followed by R2 (35.29%) and R1 (14.71%). The highest-risk groups, R4 and R5, were rare (2.94% each), showing that although these genotypes are present, they remain uncommon (Table 3).

Table 3. Scrapie risk group distribution in analyzed populations

NSP	Awassi (%)	Karakachanian (%)	Ovchepolian (%)	Sharplaninian (%)
R 1		30.43	13.19	14.71
R 2	1.09	54.35	36.26	35.29
R 3	98.91	15.22	36.26	44.12
R 4			5.49	2.94
R 5			8.79	2.94

DISCUSSION

National scrapie programs have been developed in many European Union countries. As a result of these initiatives, the genomes of numerous sheep breeds have been analyzed, enabling large-scale genotyping and a better understanding of the genetic structure of different breeds with respect to scrapie. These activities facilitate the development of targeted strategies to increase the frequency of the resistant ARR allele and reduce or eliminate the highly susceptible VRQ allele. Furthermore, the knowledge gained from these programs can assist in creation of measures to conserve the genomes of rare or endangered sheep breeds.

The frequencies of PrP alleles varied considerably among the analyzed sheep populations, the most frequent allele in the Awassi breed, Ovchepolian, and Sharplaninian pramenka was ARQ, whereas the ARR allele was predominant in the Karakachanian pramenka.

Across most continental breeds, ARQ is the predominant allele, with frequencies ranging from 50.4% to 75.9% (21, 22). The ARR is typically the second most common allele, with frequencies ranging from 18.2% to 42.5% (23, 24). Exceptions have been reported in certain German sheep breeds, where ARQ was present at less than 41% in seven of sixteen breeds, and ARR exceeded 50% in five of sixteen breeds (25).

The prevalence of the ARQ allele and the low frequency of the ARR allele in our Awassi population are consistent with previous findings from studies on Awassi sheep (26, 27, 28). The very low frequency of the ARR allele in Awassi represents a significant challenge for breeding programs aimed at increasing scrapie resistance. The pronounced homozygosity of the ARQ allele (exhibiting a high level of homozygosity, 77.14%) substantially increases the genetic susceptibility of the analyzed population to scrapie. The high risk associated with this allele is particularly pronounced when the VRLQ allele is absent from the PrP gene. To improve resistance or establish a genetically resistant population of the Awassi sheep population in the country, planned mating with ARR homozygous individuals should be implemented.

In the Ovchepolian and Sharplaninian pramenka, ARQ was the predominant allele, with ARR as the second most frequent. ARR allele showed the highest distribution in the Karakachanian pramenka and the ARQ allele was second most dominant. Higher frequencies of the ARQ allele have been reported in other pramenka sheep breeds, Istrian pramenka from Slovenia (29) and Croatia (30), and Dubski pramenka (31).

In contrast to our findings, in the Greek population of the Karakachanian (Sarakatsaniko) breed, ARQ was the most common, followed by the ARR (32). In both studies, regarding the Karakachanian sheep, the frequencies of the ARH and AHQ haplotypes are less than 0.10 and there is no presence of the VRQ allele. Importance of AHQ allele is reported in Merinoland sheep, where scrapie positive heads were carriers of at least one AHQ allele (33). The AHQ allele was present only in the three native pramenka-type sheep breed at low frequencies.

Overall, ARH is considered a neutral allele, associated with low scrapie resistance when not combined with ARR (12, 34). Second most common allele in the Awassi was the ARH allele. Similar ARH distributions have been reported in Turkish Awassi population (28), although differences exist among local and improved Awassi (26). The ARH allele in the genome of native breeds had low frequencies. Substantially lower frequencies were observed in Istrian sheep (0.005) (30).

The absence of the VRQ allele in Karakachanian pramenka and Awassi genomes indicates that, in terms of PrP genetics, these breeds resemble the Lacaune and Suffolk, so-called "arginine breeds" (5). The moderate ARR frequency in Ovchepolian

and Sharplaninian pramenka provides a solid foundation for scrapie breeding programs, but the presence of VRQ in these native breeds increases their susceptibility. Notably, VRQ frequencies in Ovchepolian and Sharplaninian pramenka are higher than in Turkish and Greek native breeds (28, 32) and are comparable to Istrian pramenka (31) but lower than reported frequency in the Dubski pramenka (31).

Our study revealed 10 and 8 genotypes in the Ovchepolian and Sharplaninian pramenka, respectively, reflecting a highly informative genomic structure. The smallest number of genotypes was observed in Karakachanian pramenka and Awassi breed, five and four, respectively. The ARQ/ARQ genotype predominated only in Awassi, consistent with previous reports (26, 28), and was the second most common genotype in Ovchepolian pramenka. In the native breeds, ARR/ARQ was generally the predominant genotype, except in Sharplaninian pramenka, where resistant ARR/ARQ genotype and the less resistant ARQ/ARQ genotype share same frequency.

The genome of the Karakachanian pramenka was unique, with ARR/ARR as the second most frequent genotype. The ARR/ARH and AHQ/AHQ genotypes were exclusive to Ovchepolian pramenka, while ARH/ARH appeared only in Awassi. ARH/ARQ occurred in all breeds, being the second most frequent in Awassi, in line with earlier studies (26, 28). The highly susceptible VRQ/ARQ genotype was present only in Ovchepolian and Sharplaninian pramenka, highlighting their high risk for classical scrapie. Control breeding programs could be applied to reduce VRQ carriers in these two native populations.

The impact of VRQ allele has been documented in several studies (9, 35). In the absence of VRQ allele, scrapie can still occur in ARQ/ARQ and ARQ/ARH genotypes, but is rare in ARR/ARQ animals (9, 35). In our study, VRQ allele was absent in Karakachanian pramenka and Awassi genomes, meaning high-risk animals in these breeds belong to R3 and R2 genotype groups. The differentiation of genomes across populations is further illustrated by NSP classification. Almost the entire Awassi population belongs to R3, with only one ARR/ARQ carrier. The absence of R4 and R5 genotypes in Awassi is advantageous, but the lack of R1 and low R2 genotype frequencies are limiting factors, making some R3 group genotypes highly susceptible. These findings emphasize the need for careful design of future scrapie resistance breeding programs in Awassi. Implementing controlled breeding is challenging given the low ARR/ARR frequency reported in other studies (26, 28).

Genomic differences among native breeds are also notable in NSP group distributions. A higher proportion of Karakachanian pramenka belong to R2, followed by R1 group of genotypes. This breed has the highest R1 frequency among native breeds, indicating strong resistance. Similar to Awassi, Karakachanian pramenka lacks R4 and R5 genotypes, with the highest risk for classical scrapie concentrated in R3 genotypes. In contrast, Greek Karakachanian population (32) show R3 dominance, followed by R2, and absence of R1. Similarities in both population of Karakachanian/ Sarakatsaniko sheep can be seen in absence of R4 and R5 genotypes. Given the small population of Karakachanian pramenka in the country, selection programs targeting specific PrP genotypes may reduce genetic variability and increase the risk of inbreeding. Therefore, future breeding programs should focus on increasing population size while maintaining less sensitive genotypes.

In Ovchepolian and Sharplaninian pramenka, all five NSP groups were present, the favorable distribution of genotypes belonging to the low (R2) and medium risk (R3) groups limits the possibility of the occurrence of the classical form of the disease. The group of R1 genotypes showed almost identical frequency in both sheep populations, but this group of genotypes has a frequency half as low as the frequency in the Karakachanian pramenka. Considering genotype diversity, R4/R5 presence, complexity regarding PrP genetics, and population sizes, these two native breeds still remain at risk for scrapie, warranting clinical monitoring and genotyping. Future scrapie control breeding programs for these two native breeds and additional genotyping should incorporate experiences and practices from actively implemented programs elsewhere in order to protect population biodiversity. Additionally, the influence of PrP genotypes on productivity traits should be considered to minimize unintended selection pressures.

CONCLUSION

The genomes of the analyzed populations represent different genetic entities and therefore they require specific selection and breeding strategies with respect to PrP genetics. The most resistant allele (ARR) of the classical form of scrapie was dominant only in the Karakachanian pramenka, while the ARQ allele showed dominance in the Awassi,

Ovchepolian, and Sharplaninian pramenka. The allele associated with high susceptibility to scrapie (VRQ) was detected only in the genomes of the Ovchepolian and Sharplaninian pramenka. Genetic resistance to the disease in the Karakachanian, Ovchepolian, and Sharplaninian pramenka is regulated by the interaction of polymorphisms at all three codons, in Awassi resistance is dependent on polymorphisms at codon 171. Overall, the risk group R3 genotypes dominates in the Awassi, Ovchepolian, and Sharplaninian pramenka while R2 group in Karakachanian pramenka. The highest risk groups genotypes (R4 and R5) are limited to the Ovchepolian and Sharplaninian pramenka. Based on the results obtained, selection towards genetic resistance to scrapie should be carried out with great caution to avoid further loss of genetic variation in autochthonous breeds. Special attention should be paid to the Karakachanian and Sharplaninian pramenka, which are generally classified as endangered and at high risk of extinction.

CONFLICT OF INTEREST

The authors declare that they have no financial or non-financial conflict of interest regarding authorship and publication of this article.

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AUTHORS' CONTRIBUTION

KP conceived, conducted the study interpreted the results and wrote the manuscript, MZ contributed to finalizing the manuscript, DK and ZTP supervised the experiments and analyzed the data.

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