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## Evaluation of the genetic resistance status to classical and atypical scrapie in Karacabey merino rams

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**Abstract:** Scrapie, the oldest prion disease of sheep, has two types: classical and atypical scrapie. It was determined that some nucleotide polymorphisms in the *PrP* coding gene have affected classical and atypical scrapie susceptibility. Consequently, EU member states have established breeding programs aiming to increase genetic resistance of their flocks to classical scrapie. These breeding programs have primarily been implemented in economically important breeds. Thus, we investigated classical and atypical scrapie related *PrP* genotypes of the Karacabey merino breed, which is of great economic importance in western regions of Turkey. In relation to classical scrapie, three alleles (ARR, ARQ, and VRQ) and five genotypes (ARR/ARR, ARR/ARQ, ARQ/ARQ, ARR/VRQ, and ARQ/VRQ) were identified. Frequencies were found to be 0.280, 0.677, and 0.043 for the alleles and 0.086, 0.376, 0.452, 0.011, and 0.075 for the genotypes, respectively. In terms of atypical scrapie, four alleles (ALRR, ALRQ, AFRQ, and VLRQ) and eight genotypes (ALRR/ALRR, ALRR/AFRQ, ALRR/ALRQ, ALRQ/AFRQ, ALRQ/ALRQ, VLRQ/ALRR, VLRQ/ALRQ, and VLRQ/AFRQ) were identified. Frequencies were found to be 0.272, 0.636, 0.049, and 0.043 for the alleles and 0.076, 0.033, 0.348, 0.043, 0.413, 0.011, 0.054, and 0.022 for the genotypes, respectively. Three nonsynonymous and two silent additional polymorphisms were also determined along with the *PrP* coding gene.

**Key words:** Karacabey merino, classical scrapie, atypical scrapie, *PrP* gene, polymorphisms

### 1. Introduction

After a modification in its three-dimensional structure, a normal host-encoded prion protein is converted into an abnormal isoform that is termed a prion. Prions cause transmissible spongiform encephalopathies (TSEs), which are fatal neurodegenerative disorders such as Gerstmann–Straussler–Scheinker (GSS) syndrome and Creutzfeldt–Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in small ruminants (1).

Scrapie is the oldest prion disease of sheep, known about for 250 years, and therefore regarded as the prototype of other TSEs (2). The disease is characterized by long incubation periods from months to years, progressive vacuolization, and amyloid plaque formation in the central nervous system (3). It is thought that the cattle prion disease BSE, which can be transmitted to humans and causes a new variant of CJD (4), originated from the use of the scrapie-contaminated products in cattle nutrition (5).

It is well described that the prion protein gene (*PrP*) modulates susceptibility to classical scrapie.

Polymorphisms at codons 136, 154, and 171 of the *PrP* have strong influence on the development of the disease in sheep (6). According to the *PrP* genotype, classical scrapie risk groups from R1 (at lowest risk) to R5 (at highest risk) were designated in the UK in 2001 (7), and several EU member states and the USA have adopted selective breeding programs aiming to increase genetic resistance to scrapie (8).

In 1998, an atypical type of scrapie was diagnosed in Norway. Its histopathological features were different from those of classical scrapie and it was termed Nor98 (9). In the following years, many atypical cases were reported, especially in European states (10,11). Further studies have shown that some *PrP* variants are also related to atypical scrapie susceptibility (12,13).

The Karacabey merino, one of the major breeds of the sheep industry in Turkey, was developed by crossbreeding German mutton-wool merino rams with native Kivircik ewes in the 1930s (14). The breed has adapted well, especially to the South and North Marmara as well as the West Anatolia regions of Turkey. Purebred Karacabey

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Merino sheep have been raised at the Bandırma Sheep Research Institute and breeding rams and ewes sold to herders who want to improve the mutton characteristic of their flocks.

Because genotypic data are essential to establish breeding programs for resistance to scrapie, this research was carried out to investigate and evaluate classical and atypical scrapie resistance status in Karacabey merino rams. Many studies have been conducted in order to genotype *PrP* polymorphisms in almost all native Turkish sheep breeds but not the Karacabey merino; therefore, this is the first *PrP* genotyping study in this breed.

## 2. Materials and methods

### 2.1. Animals

Ninety-three purebred healthy Karacabey merino rams ranging from 1.5 to 4 years of age were selected for *PrP* genotyping. Under aseptic conditions, 10-mL whole blood samples were taken from the jugular vein in sterilized tubes with EDTA and the samples were stored at  $-20^{\circ}\text{C}$  in a freezer until laboratory work.

### 2.2. DNA extraction and polymerase chain reaction (PCR)

DNA extraction was conducted using commercial kits (Qiaamp DNA Blood Mini kit); through to the isolation process the instructions of the manufacturer were followed. In order to amplify the coding region of the sheep *PrP* gene (Gen-Bank accession number AF195247), a polymerase chain reaction (PCR) (Labnet International, Inc.) was performed using previously described primers (15) and an approximately 750 bp long DNA fragment was amplified. The reaction mixture contained 1 U of Taq polymerase (Thermo Scientific), 100  $\mu\text{M}$  of dNTP (Thermo Scientific), 2.5 mM of  $\text{MgCl}_2$ , 2.5 mM of 10  $\times$  PCR buffer (Thermo Scientific), 50–100 ng of genomic DNA, 10 pmol of each of the primers, and  $\text{dH}_2\text{O}$  to a final volume of 25  $\mu\text{L}$ . The reaction conditions were as follows: 94  $^{\circ}\text{C}$  for 2 min; 30 cycles of 94  $^{\circ}\text{C}$  for 1 min, 57  $^{\circ}\text{C}$  for 1 min, and 72  $^{\circ}\text{C}$  for 1 min; and a final extension at 72  $^{\circ}\text{C}$  for 10 min. PCR products were checked on 2% agarose

gel. DNA isolation and PCR reactions were performed in the molecular genetics laboratory of the Bandırma Sheep Research Institute.

### 2.3. Sequence analysis

Sequence analyzing was carried out in Iontek laboratories (Iontek Inc., İstanbul, Turkey) using an Applied Biosystems ABI 3100 Genetic Analyzer. Observation of the nucleotide changes on the chromatograms was performed with FinchTV software version 1.4.0 (<http://www.geospiza.com/ftvdlinfo.html>). Sequences were aligned and compared using MEGA software version 6 (16). All sequences were compared with reference sequences (GenBank accession number AF195247) and all nucleotide changes along with the chromatogram were noted.

### 2.4. Statistical analysis

Allele and genotype frequencies were calculated by direct counting. The calculation formula was as follows:

$$f = nij/N,$$

where *nij* is the number of animals that have the *ij* allele/genotype, and *N* is the total number of animals studied.

To see whether the distribution of the genotypes was compatible with the Hardy–Weinberg equilibrium, a chi-squared test ( $\chi^2$ ) was performed using the PopGene population genetic analysis software (17).

## 3. Results

According to codons 136, 154 and 171, which are related to classical scrapie susceptibility, three alleles, namely  $A_{136}R_{154}R_{171}$ ,  $A_{136}R_{154}Q_{171}$ , and  $V_{136}R_{154}Q_{171}$ , were present in the *PrP* gene of the Karacabey merino rams. Allele frequencies were 0.280, 0.677, and 0.043, respectively. In combination of these three alleles, five genotypes, namely ARR/ARR, ARR/ARQ, ARQ/ARQ, ARR/VRQ, and ARQ/VRQ, were identified, and genotype frequencies were found to be 0.086, 0.376, 0.452, 0.011 and 0.075 respectively. For all genotypes, there was no deviation from the Hardy–Weinberg equilibrium ( $P < 0.05$ ). Allele and genotype frequencies as well as their allocation into risk groups are given in Table 1.

**Table 1.** Classical scrapie related allele/genotype frequencies and allocation of the genotypes into risk groups.

Alleles	<i>n</i>	Frequencies	Genotypes	<i>n</i>	Frequencies	Risk groups
ARR	52	0.280	ARR/ARR	8	0.086	R1
ARQ	126	0.677	ARR/ARQ	35	0.376	R2
VRQ	8	0.043	ARQ/ARQ	42	0.452	R3
			ARR/VRQ	1	0.011	R4
			ARQ/VRQ	7	0.075	R5

When considering atypical scrapie susceptibility status and including codon 141, four alleles, namely AL<sub>141</sub>RR, AL<sub>141</sub>RQ, AF<sub>141</sub>RQ, and VL<sub>141</sub>RQ, were present and allele frequencies were 0.280, 0.629, 0.048, and 0.043, respectively. Atypical scrapie related genotypes were as follows: ALRR/ALRQ, ALRQ/ALRQ, ALRR/VLRQ, ALRQ/VLRQ, ALRR/ALRR, AFRQ/VLRQ, ALRR/AFRQ, and AFRQ/ALRQ. With reference to risk levels, genotype frequencies were 0.817 for group 1, 0.086 for group 2, 0.022 for group 3, and 0.075 for group 4. Atypical scrapie related genotypes and frequencies are presented in Table 2.

We also examined the regions aside from standard codons and found five additional polymorphisms and determined their distributions according to *PrP* genotypes; they are shown in Table 3.

#### 4. Discussion

As seen in Table 1, the most frequent allele is the wild-type ARQ and in connection therewith ARQ/ARQ is the most common genotype in Karacabey merino rams. While the ARR allele, related to high resistance, is present at 28%,

the VRQ allele, which is the most susceptible one, is only present at 0.43%. These data are compatible with those obtained from other Turkish native sheep breeds (15,18–21). With respect to their relevance to disease resistance/susceptibility, *PrP* genotypes were classified into five groups from the most resistant (R1) to the most susceptible (R5) (7), as presented in Table 1. The frequencies of the risk groups, which are accepted as genetically resistant and/or most resistant (R2 + R1), appeared to be 46.2%, while that of the R3 group, which has little resistance, appeared to be 45.2%. The genetically susceptible and/or most susceptible groups (R4 + R5) have only 8.6% frequency. Our results suggest that the Karacabey merino can be classified in the R3 to R1 risk groups, which have little to high resistance against classical scrapie.

Recent studies revealed that polymorphisms of codons 141 and 154 are correlated with atypical scrapie development (12,22); moreover, in a matched case-control study, atypical scrapie related genotypes were classified into five risk groups (group 1 to group 5) according to their odds ratio (13). Classification of the Karacabey

**Table 2.** Atypical scrapie related allele and genotype frequencies of the *PrP* gene.

Alleles	<i>n</i>	Frequencies	Genotypes	<i>n</i>	Frequencies	Risk groups
ALRR	52	0.280	ALRR/ALRQ	32	0.817	1
ALRQ	117	0.629	ALRQ/ALRQ	38		
AFRQ	9	0.048	ALRR/VLRQ	1		
VLRQ	8	0.043	ALRQ/VLRQ	5		
			ALRR/ALRR	8	0.086	2
			AFRQ/VLRQ	2	0.022	3
			ALRR/AFRQ	3	0.075	4
			AFRQ/ALRQ	4		
			-			5

**Table 3.** The distribution of additional polymorphisms according to classical scrapie related *PrP* genotypes.

Amino acid substitutions	Genotypes				
	ARR/ARR (n)	ARR/ARQ (n)	ARQ/ARQ (n)	ARR/VRQ (n)	ARQ/VRQ (n)
Q101R	-	-	3	-	-
H143R	-	7	35	-	4
R231R	-	1	3	-	-
L237L	-	-	2	-	-
P241S	-	2	8	-	-

merino *PrP* genotype with reference to atypical scrapie risk levels is given in Table 2. Approximately 82% of the rams studied are in group 1 with minimum risk level and no animal is in risk group 5, which is estimated to be under the highest risk. It seems that Karacabey merino rams are at minimal risk in terms of atypical scrapie. In another study atypical scrapie resistance was examined in native Turkish sheep breeds. According to the results of that study, native Turkish sheep breeds are safe when considering atypical scrapie risk assessment (23) as in the Karacabey merino.

We also determined five additional polymorphisms; three of them were nonsynonymous additional changes, whereas amino acid substitutions were Glutamine (Q)→Arginine (R) at codon 101, Histidine (H)→Arginine (R) at codon 143, and Proline (P)→Serine (S) at codon 241, and two of them were silent polymorphisms at codons 231 and 237. Additional polymorphisms and their distribution according to genotypes are given in Table 3. Four additional polymorphisms we determined were previously reported in Turkish native sheep, but, to our knowledge, P241S is reported for the first time from Turkish breeds in our study.

Neither classical nor atypical scrapie has ever been reported in Turkey, but especially classical scrapie has been reported in some countries neighboring Turkey, such as Bulgaria (24), Greece (25), and even the Turkish Republic of Northern Cyprus (26). Furthermore, a 47-year-old Turkish man who had never lived abroad was diagnosed with vCJD (27). Considering the epidemiological connection between scrapie, BSE, and human vCJD as well as the fact that Turkey has imported sheep from some European States in different years, this vCJD case implies

that the presence of scrapie and/or BSE in livestock in Turkey is possible.

A regulation issued by the European Union (EU) Commission in 2003 required member states to establish breeding programs to select for resistance to TSE in each of its sheep breeds. This plan is based on elimination of the most susceptible allele (VRQ) and increasing the most resistant allele (ARR) frequencies (28). It is predicted that a breeding program for resistance to scrapie based on genotyping only rams is the most cost effective way (29), and genotyping of rams used in pure breeding has been proposed as one of the most effective strategies (30).

In conclusion, we think that it is necessary to establish breeding programs in terms of increasing resistant alleles in Turkish sheep breeds as well. As a matter of priority, these programs should first involve rams of the economically important purebred flocks such as the Karacabey merino and then be expanded toward base herds. The results of the present research show that the Karacabey merino has sufficient ARR allele frequencies to implement selective breeding programs against classical scrapie and the presence of quite lower VRQ allele frequencies will be an advantage for such programs. Furthermore, because the majority of studied rams (82%) were allocated into group 1 with minimum risk level, the risk of atypical scrapie may be negligible for the Karacabey merino.

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