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Prion protein gene (PrP) polymorphisms in healthy sheep in Turkey

Y. Oner[1†], K. Yesilbag[2], E. Tuncel[1] and C. Elmaci[1]

[1]*Department of Animal Science, Faculty of Agriculture, Uludag University, Bursa 16059, Turkey;* [2]*Department of Microbiology, Faculty of Veterinary Medicine, Uludag University, Bursa 16059, Turkey*

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Scrapie, a transmissible spongiform encephalopathy (TSE) or prion disease, is a fatal, neurodegenerative disease in sheep and goats. This disease has been known in Europe for more than 250 years. Susceptibility to scrapie is associated with polymorphisms in the sheep prion protein gene (PrP) gene. In sheep, polymorphism in the PrP gene has been identified at a number of codons, and polymorphisms at codons 136, 154 and 171 have reported linkage with susceptibility to scrapie. Polymorphisms at the PrP locus were studied in 413 animals representing three native sheep breeds (Imroz, Chios and Kıvırcık) in Turkey. Genomic DNA was obtained from blood, and genotypes were screened using PCR and direct DNA sequencing. We report 17 genotypes derived from seven different alleles. The most frequent genotype in the Kıvırcık sheep is ARQ/ARQ, whereas the ARR/ARQ genotype is predominant in the Chios and Imroz breeds. In general, the ARQ haplotype was the predominant haplotype. ARQ haplotype was also predominant in the Kıvırcık and Chios sheep breeds, whereas the Imroz sheep predominantly had the ARR haplotype. The susceptibility-associated VRQ haplotype was found in 2.38%, 0.35% and 0.81% of the Imroz, Kıvırcık and Chios sheep, respectively. Moreover, seven additional polymorphisms have been detected at codons G127S, G127V, H143R, G145S, Y172D, N174Y and Q189L. Among these polymorphisms, the N174Y allele is a novel polymorphism, and the G145S allele is a novel allele for a known polymorphic locus.

Keywords: sheep, scrapie, PrP, polymorphism, susceptibility

Implications

Scrapie in sheep is a transmissible spongiform encephalopathy present in most sheep-producing countries. Susceptibility of sheep to scrapie infections is known to be modulated by the prion protein gene (PrP) genotype of the animal. It means that certain sheep are more resistant than others to developing scrapie. Genotyping for the PrP gene at these codons is a key element of the Scrapie Eradication Program. This study is the largest study with regard to the structure of genotype profile of Turkish sheep breeds and it will contribute to design the National Scrapie Eradication Program.

Introduction

Prions are proteins that play a central role in transmissible spongiform encephalopathy (TSE) in a variety of mammals (Heaton *et al*., 2003). Scrapie is a fatal, neurodegenerative disease that affects sheep and goats. It belongs to a group of disorders known as TSE, or prion disease. The primary cause of this disease is a post-translational change in the conformation of a host-encoded cellular prion protein (PrP^{C}) to a protease-resistant isoform (PrP^{Sc}; Prusiner, 1991).

In sheep, the prion protein gene (PrP) gene is located on chromosome 13 and consists of three exons of 52, 98 and 4028 base pairs (bp), separated by two introns of 2421 and 14 031 bp. Its open reading frame (ORF) is located on exon three, and the resulting protein is 256 amino acids long (Goldmann *et al*., 1990; Lee *et al*., 1998; Tranulis, 2002). Until now, several polymorphisms have been described in the sheep PrP gene (Goldmann *et al*., 1990 and 2005; Gonzalez *et al*., 2002). In sheep, susceptibility to scrapie has been shown to be associated with five haplotype alleles (ARR, AHQ, ARH, ARQ and VRQ) of the PrP gene that result from polymorphisms at codon 136, 154 and 171 (Goldmann *et al*., 1994; Hunter *et al*., 1996). Depending on the haplotype combinations of these three codons, a total of 15 allelic combinations associated with different levels of susceptibility to scrapie have been described. According to different levels of susceptibility to scrapie, the PrP genotypes were

† E-mail: yaseminoner@yahoo.com

Table 1 *Sheep populations sampled in this study*

Breed	Population number and source	Sample size
Imroz (Gökçeada)	Two populations of Gökçeada in Çanakkale	147
Kıvırcık	Two populations of Orhangazi and Orhaneli in Bursa	142
Chios (Sakız)	Three populations of Çeşme in İzmir	124
Total		413

classified into five risk groups (R1 to R5). The R1 genotypes are associated with very low risks for scrapie, and the R5 genotypes are associated with high susceptibility to the disease (Dawson *et al*., 1998; Scrapie-Advisory notes, 2004). Studies of polymorphisms at codons 136, 154 and 171 for different breeds from a number of countries have shown that the ARR haplotype is associated with resistance to scrapie. However, the VRQ haplotype has been associated with higher susceptibility to scrapie (Belt *et al*., 1995; Hunter *et al*., 1996; Drögemüller *et al*., 2001).

Turkey is a scrapie-free country and there have been no previous reported cases of scrapie. The aim of this study was to investigate variations of the PrP gene in healthy Turkish sheep breeds and contribute to the genetic scrapie risk assessment. From the frequency distribution of PrP genotypes, the risk of scrapie in Turkish sheep was estimated.

Material and methods

Sheep and DNA sources

A total of 413 (Table 1) unrelated sheep from three native Turkish breeds were randomly sampled from their original region (Figure 1). From each animal, EDTA-treated blood (Venoject tubes, Terumo Europe, Leuven, Belgium) was collected for genotyping. No clinical signs of scrapie were ever observed in the animals of the examined flocks.

DNA extraction and amplification

Genomic DNA was isolated from the whole EDTA-treated blood (Venoject tubes, Terumo Europe) using a Genomic DNA Purification kit for mammalian blood (K0512, Fermentas, Vilnius, Lithuania) according to the manufacturer's protocols.

A 356-bp fragment of the ORF of the PrP gene including codons 136, 154 and 171 was amplified by PCR with forward (5′ TCA AGG TGG TAG CCA CAG TCA GT 3′) and reverse (5′ CCA CTC GCT CCA TTA TCT TGA TGT 3′) primers according to Gama *et al*. (2006). The amplification reactions were carried out on a TechGene (Techne, Cambridge, UK) thermal cycler in 25 μl reactions containing 50 to 100 ng genomic DNA, 2X PCR Master Mix (Fermentas, Vilnius, Lithuania) and 0.5 mM of each of the primers. The temperature profile of the PCR was programmed for 35 cycles of 30 s at 95°C, 45 s at 59.3°C and 30 s at 72°C. Finally, an extension step of 30 min at 72°C was performed. The PCR products were visualized by staining with ethidium bromide after electrophoresis of 10 μl of the reaction mixture on a 2% agarose gel.

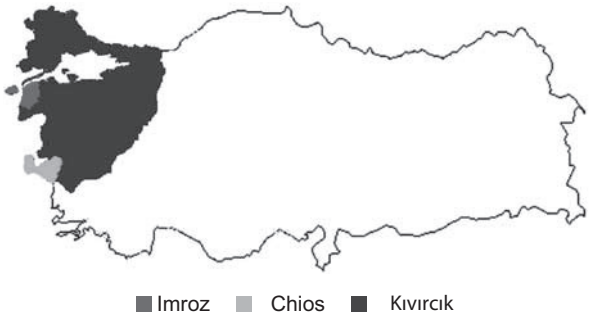

Figure 1 Geographic distribution of sampled sheep breeds.

PCR cleanup and sequencing

DNA samples were sequenced with Applied Biosystems Taq Dyedeoxy Terminator cycle sequencing kits, which use the fluorescently labeled dideoxynucleotide chain termination method. After cycling and clean up, the DNA samples were then resolved by capillary electrophoresis on an ABI 3730XL DNA Analyzer, which translates the fluorescent signals into their corresponding nucleotide sequence. Before sequencing, the PCR products were purified using PCR Product Purification Kit (Roche, High Pure PCR Product Purification Kit). The forward PCR primer was used to sequence the PCR reaction.

Statistical analysis

Genotype, allele and haplotype frequencies for each population were calculated using the PopGene population genetic analysis software (Yeh *et al*., 2000). This software program was also used to carry out a statistical test to determine possible deviations from the Hardy–Weinberg equilibrium.

Results

PrP allele distribution

A total of 413 sheep belonging to three sheep breeds were studied with regard to PrP gene polymorphisms. PrP allele frequencies for each breed are shown in Table 2. The polymorphisms detected in the prion gene make up seven allelic variants (ARR, AHQ, ARH, ARQ, VRQ, ARK and TRQ). Some differences between breeds were observed. In addition to the five well-known alleles (ARR, AHQ, ARH, ARQ and VRQ), two rare alleles (ARK and TRQ) were found in sheep breeds investigated. ARQ, the allele associated with susceptibility to scrapie, was most frequent in the Kıvırcık sheep breed. The ARR allele, which is relatively resistant, was found at high frequency in Imroz sheep. The AHQ allele was found in all

Table 2 *Allele haplotype frequencies at the PrP locus studied sheep breeds*

		Allele haplotype (%)						
Breed	*n*	ARR	AHQ	ARH	ARQ	VRQ	ARK	TRQ
Imroz	147	147	17	5	118	7	–	–
		50	5.78	1.70	40.14	2.38		
Kıvırcık	142	49	16	56	161	1	1	–
		17.25	5.63	19.72	56.69	0.35	0.35	
Chios	124	76	17	44	98	2	–	11
		30.64	6.85	17.74	39.52	0.81		4.44
Total	413	272	50	105	377	10	1	11
		32.93	6.05	12.71	45.64	1.21	0.12	1.33

Prp = prion protein gene.

three breeds at a low frequency. The ARH allele was detected at a relatively high frequency in the Kıvırcık and Chios sheep breeds, but at a low frequency in the Imroz sheep. The VRQ allele, which is associated with the highest susceptibility to scrapie, was detected at low frequencies in all three breeds. The ARK allele was detected at low frequencies in only one Kıvırcık sheep in the heterozygous condition. The TRQ allele was found only in the Chios sheep breed. Overall, the more frequent alleles were ARQ (45.64%), followed by ARR (32.93%), ARH (12.71%), AHQ (6.05%) and VRQ (1.21%; Table 2).

Other PrP polymorphisms

In the Turkish sheep breeds investigated, seven additional amino acid polymorphisms were found at codons G127S, G127V, H143R, G145S, Y172D, N174Y and Q189L (Table 3). Two additional polymorphisms have been detected at the first and second nucleotide of codon 127, leading to changes from glycine to serine and valine, respectively. The change in amino acid from histidine to arginine at codon 143 is the most common change among these additional substitutions. A new mutation at codon 145 was previously reported by Álvarez *et al*. (2007). However, polymorphisms at the second nucleotide position of the codon leads to changes from glycine to valine. We detected a novel base substitution at the first nucleotide (G→A) resulting in serine. The replacement of tyrosine (TAT) for aspartic acid (GAT) at codon 172 is the result of a T→G transversion at the first nucleotide position in the codon. In codon 174, a base substitution at the first nucleotide position caused by an A→T transversion resulted in a novel change from asparagine (AAC) to tyrosine (TAC). Although the polymorphism at codon 172 has also been detected in some Spanish breeds and in the Imroz sheep breed (Acín *et al*., 2004; Un *et al*., 2008), this is the first report of the polymorphism in codon 174 in Kıvırcık sheep. The last polymorphism we detected was the A→T substitution at the second nucleotide position of codon 189, which caused a change to leucine from glutamine.

PrP genotype distribution

In total, 413 animals from three sheep breeds were genotyped at the PrP locus (Table 4). A total of 17 genotypes were found, and 13 of the 15 well-known genotypes associated with scrapie resistance were found in the sheep population investigated. Eleven well-known genotypes were identified in the Imroz breed. The most frequent genotypes were ARR/ARQ (33.33%), ARR/ARR (29.93%) and ARQ/ARQ (19.05%) in Imroz sheep breed. Similarly, ARR/ARQ (22.58%) was the most frequent genotype in the Chios sheep breed, followed by ARQ/ARQ (20.16%), ARR/ARR (15.32%) and ARH/ARH (12.10%). Overall, 13 genotypes were found in the Chios breed, of which 10 were associated with scrapie but three of them were not. There were a total of nine animals with these rare genotypes. Eleven genotypes were identified in the Kıvırcık breed and the most frequent genotypes were ARQ/ARQ (30.28%), ARR/ARQ (24.65%) and ARH/ARQ (19.01%). Only two animals (1.41%) had the most resistant ARR/ARR genotype from the Kıvırcık breed (Table 4).

Overall, the most frequent genotype was ARR/ARQ (27.12%), followed by ARQ/ARQ (23.24%) and ARR/ARR (15.74%). The ARR/ARQ genotype was predominant in Imroz and Chios sheep, and the ARQ/ARQ genotype was predominant in the Kıvırcık breed. The ARR/VRQ genotype was not found in any of the animals investigated. The VRQ/VRQ genotype, which is highly associated with susceptibility to scrapie, was found at very low frequencies; there were only two animals with this genotype from the Imroz and Chios sheep breeds. The distribution of genotypes differed from the Hardy–Weinberg equilibrium for all sheep breeds investigated.

Table 3 *Additional polymorphisms detected*

		Number of animals	
Breed	Substitution	Homozygote	Heterozygote
Kıvırcık	G127V	–	5
Kıvırcık	G127S	6	–
Kıvırcık	H143R	–	24
Kıvırcık	G145S	–	1
Kıvırcık	Y172D	–	7
Imroz	Y172D	–	3
Kıvırcık	N174Y*	1	8
Kıvırcık	Q189L	1	–

*New polymorphism.

Table 4 *The PrP genotype frequencies in sheep of this study*

	Breed							
	Imroz		Chios		Kıvırcık		Total	
Genotype	*n*	%	*n*	%	*n*	%	*n*	%
ARR/ARR	44	29.93	19	15.32	2	1.41	65	15.74
ARR/AHQ	9	6.12	2	1.61	2	1.41	13	3.15
ARR/ARH	1	0.68	8	6.45	8	5.63	17	4.12
ARR/ARQ	49	33.33	28	22.58	35	24.65	112	27.12
AHQ/AHQ	–	–	2	1.61	–	–	2	0.48
AHQ/ARH	–	–	–		3	2.11	3	0.73
AHQ/ARQ	8	5.44	9	7.26	11	7.75	28	6.78
ARH/ARH	1	0.68	15	12.10	9	6.34	25	6.05
ARH/ARQ	1	0.68	6	4.84	27	19.01	34	8.23
ARQ/ARQ	28	19.05	25	20.16	43	30.28	96	23.24
ARR/VRQ	–	–	–	–	–	–		–
VRQ/AHQ	–	–	–	–	–	–		
VRQ/ARH	1	0.68	–	–	–	–	1	0.24
VRQ/ARQ	4	2.72	–	–	1	0.70	5	1.21
VRQ/VRQ	1	0.68	1	0.81	–	–	2	0.48
ARQ/ARK	–	–	–	–	1	0.70	1	0.24
AHQ/TRQ	–	–	2	1.61	–	–	2	0.48
ARQ/TRQ	–	–	5	4.03	–	–	5	1.21
TRQ/TRQ	–	–	2	1.61	–	–	2	0.48
	147		124		142		413	

Prp = prion protein gene; *n* = number of animals.

Table 5 *PrP genotypes of this study and distributions in the risk groups*

	Breed							
	Imroz		Chios		Kıvırcık		Total	
Risk group	*n*	%	*n*	%	*n*	%	*n*	%
R1	44	29.93	19	15.32	2	1.41	65	15.74
ARR/ARR								
R2	59	40.14	38	30.64	45	31.69	142	34.38
ARR/AHQ, ARR/ARH, ARR/ARQ								
R3	38	25.85	57	45.97	93	65.49	188	45.52
AHQ/AHQ, AHQ/ARH AHQ/ARQ, ARH/ARH ARH/ARQ, ARQ/ARQ								
R4	–		–		–			
ARR/VRQ								
R5	6	4.08	1	0.81	1	0.70	8	1.94
VRQ/AHQ, VRQ/ARH VRQ/ARQ, VRQ/VRQ								
Unknown	–		9	7.26	1	0.70	10	2.42
Total	147		124		142		413	

Prp = prion protein gene.

Risk analysis for scrapie

The sheep PrP genotypes were classified into five risk groups (R1 to R5) according to different levels of susceptibility to scrapie. Genotypes in group R1 were associated with very low risks of scrapie, and genotypes in group R5 were associated with a high susceptibility to the disease (Dawson *et al.*, 1998; Scrapie-Advisory notes, 2004). For the overall population, the ARR/ARQ and ARQ/ARQ genotypes accounting for the highest frequency in investigated sheep breeds belonged to the R2 and R3 risk groups, respectively. The most resistant ARR/ARR genotype was present in 29.93% of Imroz sheep, 15.32% of Chios sheep and 1.41% of Kıvırcık sheep (Table 5). The high-risk groups of R4 and R5 are combined as VRQ-carrying genotypes appeared at very low frequencies (Table 5).

Discussion

Scrapie has never been detected in native Turkish sheep breeds. Although PrP polymorphisms associated with resistance or susceptibility to scrapie were investigated in many sheep breeds from all over the world, information on the presence or frequency of these polymorphisms in Turkish sheep breeds is very limited. In this study, resistance or susceptibility of three Turkish sheep breeds to scrapie has been investigated.

Although the ARR/VRQ genotype was not found in any of the breeds investigated in this study, Un *et al*. (2008) detected this genotype in the Kıvırcık and Chios breeds at low frequency. The susceptibility-associated VRQ/VRQ genotype was found at very low frequencies; there were only two animals from the Chios and Imroz breeds with this genotype. In contrast to our findings, Un *et al*. (2008) did not find any VRQ homozygote animals in these sheep breeds. In addition, Lühken *et al*. (2008) investigated four native Turkish sheep breeds for PrP gene polymorphisms, and also did not find any homozygote VRQ animals among these four breeds, although they found VRQ heterozygote animals at very low frequency in the Dağlıç sheep breed. These inconsistencies can be attributed to the limited number of animals used in previous studies.

In this study, alanine, valine and threonine alleles were found at codon 136. The alanine allele was the most common allele, and valine and threonine alleles were rare. Although the threonine allele was found only in the Chios breed in this study, Un *et al*. (2008) found the same allele in both Chios and Kıvırcık sheep in Turkey. There were only two alleles, arginine and histidine, at codon 154. At codon 171, there were glutamine, histidine, arginine and lysine alleles, and the lysine allele was found only in Kıvırcık sheep. The same allele was reported in Mongolian (Gombojav *et al*., 2003), Awassi (Gootwine *et al*., 2008), Greek (Billinis *et al*., 2004) and Bergamasca sheep (Lühken *et al*., 2008) at low frequencies. This study showed that all of the common alleles observed so far in the other sheep breeds were also found in the investigated Turkish sheep.

As a result of this study, five common previously reported haplotype alleles (ARR, AHQ, ARH, ARQ and VRQ) and two rare alleles (ARK and TRQ) were found in three native Turkish sheep breeds. Interestingly, the TRQ allele was observed only in Chios sheep, whereas the ARK allele was found in Kıvırcık sheep. Although this ARK rare haplotype allele was found together with the ARH allele by Acutis *et al*. (2006) in one scrapie-positive sheep, in this study we found it together with ARQ, as was described by Portela *et al.* (2006). Gootwine *et al*. (2008) also detected the ARK allele in the Awassi breed of sheep, but only as homozygotes. The frequency of the ARK allele was calculated as 0.04098 and 0.003 among Assaf and Awassi sheep breeds by Portela *et al.* (2006) and Gootwine *et al*. (2008), respectively. The association with susceptibility to scrapie of this rare allele has not yet been clarified.

The other rare haplotype allele, TRQ, was found only in the Chios breed in this study, similar to what has been reported for the Chios breed reared in Greece (Billinis *et al*., 2004). Un *et al*. (2008) also found this allele in the Chios breed, but they also found it in the Kıvırcık breed.

The ARR allele was observed in all breeds, but the frequencies were different, ranging from 17.25% in Kıvırcık sheep to 50% in Imroz sheep. In Chios sheep, the frequency of the ARR allele was 30.64%. The most frequent allele was ARQ (45.65%), and it is believed to be a wild-type allele. The genotypes with more than 15% frequency were ARR/ARQ, ARQ/ARQ and ARR/ARR (27.12%, 23.24% and 15.74%, respectively) in this study. The VRQ allele, which is known to be associated with high susceptibility to scrapie, was found in only one Kıvırcık sheep, two Chios sheep and seven Imroz sheep. The overall frequency of the VRQ allele was observed to be 1.21% in the investigated sheep. The frequency of the AHQ allele was 6.05%. These results are in accordance with several investigations, which found that the ARQ allele was the most frequent, followed by the ARR allele. These results were found in Austrian sheep (Sipos *et al*., 2002), Germen sheep (Lühken *et al*., 2004), Spanish sheep (Acín *et al*., 2004; Ponz *et al*., 2006), Portuguese sheep (Gama *et al*., 2006), Awassi and Assaf sheep in Israel (Gootwine *et al*., 2008), Turkish sheep breeds (Un *et al*., 2008) and in Istrian and crossbred sheep (Cubric-Curic *et al*., 2009).

In addition to the polymorphisms known to be related with classical scrapie, numerous new polymorphisms have been reported in different codons in our study, similar to the others (Thorgeisdottir *et al*., 1999; Vaccari *et al*., 2001; Wang *et al*., 2008). Most of the additional polymorphisms detected in Kıvırcık sheep are similar to those detected in the study carried out by Un *et al*. (2008). Despite this, these additional polymorphisms were detected at different loci. These findings support the hypothesis that among these three native Turkish sheep breeds, the Kıvırcık breed has the highest variability. This breed may be useful to research the relationships between new polymorphisms and susceptibility and resistance to different forms of scrapie that currently remain unknown.

The results showed that when classified into risk groups, the majority of the investigated sheep belonged to group R3 (45.52%), followed by R2 (34.38%) and R1 (15.74%). In all, eight animals of the 413 studied carried genotypes included in the R5 group that are associated with high susceptibility to scrapie. The majority of the animals investigated were grouped in R3 and R2. The R3 group should be examined when these animals are used in breeding programs. On the other hand, in order to avoid scrapie disease among sheep populations, genotyping these PrP gene codons should be included in breeding schemes. The data from our study may provide preliminary information for selecting scrapie-resistant sheep for breeding strategies.

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