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Polymorphisms of ovine prion protein (PrP) gene in Pramenka sheep breed population in Bosnia and Herzegovina

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Abstract: Transmissible spongiform encephalopathies (TSEs) are a group of diseases that affect the nervous system and lead to death. The putative infectious agent is the host-encoded prion protein (PrP) and it appears that the development of scrapie in sheep is closely related to polymorphisms in the host PrP gene. The aim of this study was to investigate three well-known polymorphisms in exon 3 of the PrP gene (at codons 136, 154, and 171) that have a clear and significant effect on scrapie in sheep. Four other polymorphisms (at codons 145, 185, 231, and 237) that are yet undefined were also investigated. The study was performed on the Bosnian Pramenka breed of sheep. Genomic DNA was isolated from venous blood and then a 628 bp long DNA fragment from exon 3 was amplified using the PCR method. The results from this study indicate the presence of seven different genotypes associated with susceptibility to scrapie in the investigated Bosnian sheep population, of which four haplotypes were proven to be susceptible to developing the disease. Two of them were at codon 231 and 237 and have not been described before.

Key words: PrP, transmissible spongiform encephalopathy, scrapie, sheep, polymorphism

1. Introduction

Scrapie is a prion-associated transmissible spongiform encephalopathy (TSE) that occurs in sheep and goats. Scrapie is found in most breeds of sheep, but there are differences between breeds, strains, and individuals in susceptibility to it. It has been found that the PrP gene is responsible for disease susceptibility and pathogenesis. The sheep PrP gene consists of three exons and two introns and is located on chromosome 13 (1), a region considered to be evolutionarily preserved (2). In general, the PrP gene is located in the promoter coding region, but in different areas in different species. The sequence of this gene was determined in 1998 and a high similarity in coding and noncoding sequences in sheep and humans was found, indicating that the two sequences are conserved in these two species (2). The ovine PrP gene is 31,412 bp long. In sheep, the genetic variability of the prion protein gene coding sequences is considered to be the most important in susceptibility to scrapie. Seven polymorphic loci (codons) were described, but the most important and significant in terms of disease expression are loci-codons 136 (valine–alanine), 154 (histidine–arginine), and 171 (glutamine–arginine). The allelic variants (haplotypes) found on these loci (codons) are ARR, ARH, AHQ, ARQ, and VRQ (3). These alleles are associated with different levels of scrapie susceptibility. The five mentioned allelic variants (haplotypes) were tested the most, and it was found that the ARR haplotype (allelic variant) was always connected with partial or complete protection from disease. Based on this information, all farming selection programs must have the aim to increase the frequency of these alleles in the population.

Different sheep genotypes are classified in groups related to their susceptibility level (see Table 1). The highest level of resistance to scrapie is provided by the ARR/ARR genotype belonging to the NSP1 group, while highly susceptible genotypes are in the NSP5 group, namely ARQ/VRQ, VRQ/VRQ, VRQ/ARH, VRQ/AHQ, and ARQ/ARR.

The aim of this study was to investigate three well-known polymorphisms in exon 3 of the PrP gene (at codons 136, 154, and 171) that have a clear and significant effect on scrapie in sheep. We also wanted to investigate four other polymorphisms (at codons 145, 185, 231, and 237), since new mutations at codons 145 (4), 185 (5), 231, and 237 (6) (7) were previously reported.

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Table 1. PrP genotype classification, based on scrapie susceptibility (3).

Classification	Genotype	Resistance
NSP1	ARR/ARR	Highly resistant
	ARR/ARQ	
NSP2	ARR/ARH	Resistant
	ARR/AHQ	
	ARQ/ARQ	
	ARQ/AHQ	
NSP3	AHQ/AHQ	Low resistance
	ARH/ARH	
	ARQ/ARH	
NSP4	ARR/VRQ	Susceptible
	ARQ/VRQ	
	VRQ/VRQ	
NSP5	VRQ/ARH	Highly susceptible
	VRQ/AHQ	
	VRQ/ARR	

2. Material and methods

Our research was conducted on the Pramenka sheep breed, Vlasic (Dubski) strain, of Bosnia and Herzegovina. The Dubski Pramenka sheep originate from the village Dub, in the Travnik area, and they are mostly farmed in central Bosnia and Herzegovina, particularly on Vlasic Mountain. An accurate number of individuals of the Dubski sheep breed is not known; however, it is certainly present in the largest numbers as compared with other sheep breeds in Bosnia and Herzegovina (8). Blood samples were collected from 42 randomly chosen healthy animals belonging to five flocks from livestock sheep farms on Vlasic Mountain (near the village of Dub). Venous blood (approx. 3 mL) was taken from the jugular vein of each individual animal and collected in sterile 3 mL K3E BD Vacutainer tubes (BD Vacutainer System, Plymouth, UK) containing ethylene diamine tetra acetic acid (EDTA), and then transported to the laboratory where they were stored at –30 °C until needed for DNA isolation. Genomic DNA was extracted from the blood samples (200 µL) using Sigma's GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich Chemie, GmbH, Germany) according to the manufacturer's instructions. The concentration of the extracted DNA was measured by a spectrophotometer (Genova, JENWAY, UK), whereas the quality of DNA was checked by gel electrophoresis (at 120 V for 45 min) on 1% TBE-agarose gel stained with ethidium bromide (EtBr).

The PrP gene fragment size of 628 bp from exon 3 was amplified using the following primers (9):

Forward 5´ – CCGCTATCCACCTCAGGGA – 3´

Reverse 5´ – TTGCCCCTATCCTACTATGAGA – 3´.

The optimization of PCR conditions by a temperature gradient PCR was carried out using an iCycler thermal cycler (Bio-Rad, Germany). Thermocycling was performed by initial denaturation for 8 min at 95 °C, followed by 34 cycles of 1 min at 94 °C, 1 min at 64 °C annealing temperature and 1 min extension at 72 °C. A final extension was performed for 7 min at 72 °C. The amplification reactions were carried out in a total volume of 20 µL containing 4 µL of DNA, 1 µL of each primer (10 pmol/µL), and 10 µL of ready-to-use GoTaq Green master mix (Promega), in an iCycler thermal cycler (Bio-Rad, Germany).

The PCR products (20 µL) were analyzed by electrophoresis (at 80 V for 30 min) on 0.8% agarose gel stained by ethidium bromide (EtBr). The visualization was performed in a UV transilluminator and the images were captured with a video documentation system (UVITEC). Amplified fragments of the appropriate size (628 bp) were cut directly from the gel and purified by Promega Wizard SV Gel and OCR Clean-Up System kits following the supplier`s instructions. Then, the amplified products

were sequenced by the standard Sanger dideoxynucleotide method, using the same primers as for the PCR reaction. The sequencing was performed on an ABI PRISM 3100-Avant genetic analyzer.

The sequences were analyzed using BioEdit software, version 7.0.9.0. (10), while the ClustalW program, version 2.2.10. (11) was used for multiple sequences alignment.

In this study, polymorphisms at loci (codons) 136, 154, 171, 145, 185, 231, and 237 were investigated in the Bosnian Pramenka sheep breed, Vlasic (Dubski) strain from Vlasic Mountain (near the village of Dub). First, the allelic variants (haplotypes) in codons (loci) 136, 154, and 171 for the SSHL ("scrapie susceptible haplotype locus") were identified and estimated using the Bayesian approach, and then the analysis and estimation of polymorphisms, allele frequencies, and genotypes were carried out at loci (codons) 145, 185, 231, and 237. The PROC haplotype and PROC allele procedures, implemented in the SAS/Genetics 9.1.3. software package (12), were used for all calculations.

3. Results

Seven genotypes on scrapie susceptible codons were found in the studied Bosnian population of sheep (Table 2). The most frequent genotype, with 47.62% of frequency appearance, was ARQ/ARQ, belonging to risk group 3 (NSP3–low resistance), while genotypes ARR/AHQ and ARR/ARQ, which belong to risk group 2 (NSP2–resistant), and genotype ARR/VRQ that belongs to risk group 4 (NSP–susceptible) were found with a frequency of 4.76%. Genotype ARR/ARR (risk group 1; NSP1–highly resistant) was present with a frequency of 9.52%.

The most frequent haplotype was ARQ with a frequency of 64.29%, while haplotype VRQ was the least frequent one, with a frequency of 7.14% (Table 3).

In this study, single nucleotide polymorphisms (SNPs) were found in all of the three investigated and well-known scrapie susceptible loci (at codons 136, 154, and 171) of the PrP gene (see Table 4).

Looking at amino acid variations at locus (codon) 136, which is responsible for the synthesis of two amino acids (alanine and valine) (6), we found that the most frequent was the alanine variant with a frequency of 92.86%, while the valine variant occurred with a frequency of 7.14% (see Table 4). The alanine variant at codon 136 is preferred due to the fact that it reduces the risk of TSE occurrence.

Considering locus (codon) 154, which is responsible for the synthesis two amino acids, arginine and histidine (6), we found that the arginine variant is more prevalent in the Bosnian population (with a frequency of 88.10%),

Table 2. Genotype frequency.

	Genotype		Risk group	Frequency (%)	n
	Codon				
136	154	171			
AA	RH	RQ	2	4.76	1
AA	RR	RQ	2	4.76	1
AV	RR	RQ	5	4.76	1
AV	RR	QQ	5	9.52	2
AA	RR	RR	1	9.52	2
AA	RH	QQ	3	19.05	4
AA	RR	QQ	3	47.62	10

Table 3. Allelic variants (haplotypes) frequency.

Allelic variants (haplotypes)	n	Frequency (%)
AHQ	5	11.90
ARQ	27	64.29
ARR	7	16.67
VRQ	3	7.14

Table 4. Polymorphism and frequency of SNPs for scrapie susceptible codons.

Codon	Allele	n	Frequency (%)
136	A_{136}GCC	39	92.86
	V_{136}GTC	3	7.14
154	H_{154}CAT	5	11.90
	R_{154}CGT	37	88.10
171	Q_{171}CAG	35	83.33
	R_{171}CGG	7	16.67

while the histidine variant was found with a frequency of 11.90% (see Table 4).

Locus (codon) 171, which is responsible for the synthesis of four amino acids (arginine, glutamine, histidine, and lysine) (7,13,14), was present in our study with only two variants: glutamine and arginine. Arginine synthesis at codon 171 forms nonrisk haplotype ARR, while the glutamine variant increases the risk of TSE. In the studied Bosnian population, arginine is represented with a frequency of 83.33%, while glutamine was with a frequency of 16.67% (Table 4). A combination of the alanine variant from codon 136 with the arginine variant of codon 171 makes the scrapie risk-free ARR haplotype.

Besides investigations on polymorphisms of those three well-known scrapie susceptible codons (loci), our examination was extended to investigations on polymorphisms of four other loci (codons 145, 185, 231, and 237) with unknown effects on the development of scrapie in sheep (unknown and undefined levels of risk) (Table 5). The absence of any polymorphisms was found at codons 145 and 185 in the Bosnian population under investigation, while silent mutations were recorded on codons 231 (AGG → CGG) and 237 (CTC→ CTG). In both cases, there were no amino acid changes found. The silent mutation that was found at locus (codon) 231, which is responsible for the synthesis of amino acid arginine, had a frequency of variant AGG 85.71%, while a variant CGG was present with a frequency of 14.29% (see Table 5). A silent mutation that was found at codon 237 (responsible for the synthesis of amino acid leucine) was present with 88.10% frequency of variant CTC and 11.90% frequency of variant CTG (Table 5).

4. Discussion

This is the first study conducted on a sheep population in Bosnia and Herzegovina regarding the genetic aspects of scrapie and PrP gene sequencing. The presence of four risk groups, according to NSP classification, was identified in our study**.** A high frequency of haplotype ARQ (64.29%) in scrapie susceptible codons was determined, which is in accordance with the values obtained in other studies (15). The ARQ haplotype could be considered as a "wild" haplotype and it is present with a high frequency in other countries as well: in Italy for the breed of Gentile di Puglia at 53.23% (15), in Austria in the Tyrolean mountain sheep at 65.7% (16), in Hungary with the breed

Table 5. Polymorphisms at undefined loci that might have some effect on disease.

Codon	Allele	Frequency (%)
145	G_{145}GGC	100.00
	S_{145}AGC	0.00
185	I_{185}ATC	100.00
	T_{185}ACC	0.00
231	R_{231}AGG	85.71
	R_{231}CGG	14.29
237	L_{237}CTC	88.10
	L_{237}CTG	11.90

of Tsigai at 53.45% (15), and in Turkey with the breeds of Imroz (40.14%), Kivircik (56.69%), Chios (39.52%) (4), Ivesi (75.00%), Morkaraman (62.16%) (17), while in the neighboring country of Croatia it is present with 67.4% in the Istrian sheep breed and with 52.6% in the crossbreeds of Istrian sheep (5). In our studied population of the Pramenka breed from Vlasic (Dubski strain), the most frequent genotype was ARQ/ARQ, which is considered to be susceptible to developing scrapie and belongs to risk group 3 (NSP3–low resistance).

The haplotype frequency of ARR (16.67%), which belongs to risk group 1 (NSP1–high resistance), was relatively high despite the fact that farming in Bosnia and Herzegovina is rather primitive, that is without systematic breed selection, particularly regarding scrapie monitoring and selection. In neighboring Croatia the presence of this ARR haplotype was found to be 22.8% in the Istrian sheep and 13.2% for hybrids (5). In the region, the frequency of the ARR haplotype was found at 35.48% in the Italian breed Gentile di Puglia; in Hungary in the breed of Tsigai it was 32.76% (15); in Turkey in the Imroz breed it was 50.00%, in the Kivircik breed 17.25%, in the Chios breed 30.64% (4), in the Ivesi breed 6.25%, and in Morkaraman breed 17.56% (17); and in Austria the frequency of this haplotype was 25.8% in the Tyrolean mountain sheep (16). An important fact is that the presence of the ARR alleles, as well as the ARR/ARR genotype, provides the opportunity to increase the resistance to scrapie in the sheep populations of Bosnia and Herzegovina if better farming practices and monitored selection are implemented in order to increase the number of animals and flocks with this favorable genotype.

The most unfavorable haplotype (VRQ) with a frequency of 7.14% in our studied sheep flocks can be considered as reasonable in comparison with other European sheep breeds, where the frequency of this haplotype ranges from 0.00% to 17.40%. For example, in the Italian sheep breed Gentile di Puglia it is 0.00% (15), in the Croatian Istrian sheep it is 1.1% and in crossbreeds of the Istrian sheep it is 0.00% (5), in Austria in the Tyrolean mountain sheep it is 0.00% (16), and in Turkey in the Imroz breed it is 2.38%, in the Kivircik breed it is 0.35%, in the Chios breed it is 0.81% (4), in the Ivesi breed 1.56%, and in the Morkaraman breed 4.05% (17), while the Hungarian Tsigai breed has a higher frequency than ours at 10.34% (15).

The polymorphisms at loci (codons) 145 and 185 were absent, while the polymorphisms at loci (codons) 231 and 237 resulted in silent mutations R → R or L → L. These polymorphisms were also found in sheep populations in neighboring Croatia (5), but the investigations of these loci (codons) were not carried out in other countries, so further comparisons are not possible at this time. The mentioned mutations occur at a rather low frequency and their impact on the occurrence of scrapie has not been determined yet.

It is important to continue the investigations and screening of other sheep flocks in Bosnia and Herzegovina, especially for seven different genotypes potentially associated with susceptibility to scrapie. It is particularly important to continue the investigation on the three proven SSHL codons (136, 154, and 171), as well as on the four additional codons investigated in our work (codons 145, 185, 231, and 237). A larger number of individuals and a larger number of loci (codons) that might have some effect on scrapie susceptibility should be included in future works. Better organized selection and breeding programs in the farms of Bosnia and Herzegovina are necessary in order to increase the ARR combination of alleles that will lead to the improvement and growth of scrapie resistant genotypes.

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