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Research article

SCRAPIE RESISTANCE GENE IDENTIFICATION USING OPTIMIZED TAQMAN TEST QPCR METHOD IN SHEEP ON THE TERRITORY OF THE REPUBLIC OF SERBIA

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Scrapie is an infectious neurodegenerative disease affecting the central nervous system of sheep and goats that belongs to transmissible spongiform encephalopathies. The disease is caused by the accumulation of proteinase-resistant isoform of the prion protein. The sheep predisposition to scrapie is associated with polymorphisms of the PrP gene. Genetic susceptibility to scrapie is mainly related to codons 136, 154, and 171. ARR sheep are strongly scrapie resistant and VRQ genotype is the most susceptible. Many countries have scrapie eradication programs based on using rams with resistant genotype. The eradication program has not yet been implemented in the Republic of Serbia. To examine the genetic makeup of sheep in Serbia related to scrapie, we optimized TaqMan probes of real-time polymerase chain reaction (qPCR) technique for three codons. Blood samples from 100 sheep were analyzed by qPCR and the majority of the examined sheep were AA homozygous for the 136 codon. For codon 154 the most frequent genotype was RR and for codon 171 the most frequent genotype was QQ.

Key words: genotyping, polymorphisms, qPCR, Scrapie, TaqMan probes

INTRODUCTION

Scrapie is a fatal, neurodegenerative disease of sheep and goats belonging to the group of transmissible spongiform encephalopathies (TSEs). As with other diseases in this group, the causative agent is a prion. Although monitoring has been conducted for years in many countries, scrapie is still endemic in many parts of the world [1]. It does not pose a direct risk to human health, but there is a hypothesis that bovine spongiform encephalopathy (BSE) in the United Kingdom in the '90s was the result of the passage of a scrapie-like agent into the cattle population [2]. Scrapie can cause serious economic

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consequences that include individual producer losses, as well as export losses that affect the entire country. Infectious prions are cellular prion proteins misfolded into an abnormal protease-resistant isoform (PrPSc) by an unknown mechanism [3,4]. Oral exposure seems to be the main route of PrPSc entry, but infection may also occur after ocular exposure or contact with injured skin or mucous membranes [5,6]. After oral exposure, PrPSc accumulates in the gut-associated lymphoid tissues (GALT) and can be detected in the tonsils, spleen, and retropharyngeal and mesenteric lymph nodes for months before reaching CNS [7]. During that time, infected sheep can shed the agent into the environment via body fluids including placental fluids, milk and colostrum resulting in vertical transmission to newborns, as well as horizontal transmission within the flock at the time of lambing [8].

The susceptibility of sheep to scrapie and the incubation period depend on polymorphism of the prion protein gene (*prnp*) that encodes the cellular protein and the nature of the prion strain. Three major sites in *prnp* associated with sheep resistance to classic scrapie are at codons 136, 154, and 171 [9]. Each allele is marked with a three-letter code and the ARR allele is associated with a highly protective effect against infection with classical scrapie but does not provide resistance towards atypical scrapie [8]. Atypical scrapie is not an infectious disease and appears sporadically as a degenerative brain disease in elderly sheep [6]. Susceptibility to classical scrapie is associated with valine (V), arginine (R) and glutamine (Q) on codons 136, 154 and 171 respectively, while alanine (A), histidine (H) and arginine (R) on the same codons provide resistance [8,10,11]. The most common haplotypes are ARR, ARQ, AHQ, ARH and VRQ [12]. The ARR haplotype is considered to have the lowest risk for classical scrapie under natural conditions. Sheep with this allele are highly unlikely to carry or transmit scrapie. However, some data suggest that ARR/ARR sheep cannot be considered fully resistant to classical scrapie, but such infections are extremely rare [9,13]. The *prnp* polymorphism in sheep has been used for years as a basis for scrapie eradication programs conducted in many countries. In the UK the scrapie disease control program by genotyping started in the 1990s. The main goal of the eradication programs is to minimize the theoretical risk of scrapie to public health using genetically most resistant rams for mating to increase the frequency of ARR alleles and reducing the frequency of risk alleles. In the long run, the program should contribute to the control and elimination of scrapie. In the USA the implementation of the scrapie eradication program had the effect of reducing the number of scrapie positive sheep at the slaughter line by 90% [8,14].

There are just a few diagnosed cases of scrapie in sheep in the Republic of Serbia. However, due to occasional outbreaks of scrapie in countries in the region, it is necessary to perform genotyping of sheep in the Republic of Serbia in order to increase the level of genetic resistance to TSEs in the national sheep flock.

MATERIAL AND METHODS

Sampling

In this study, the blood of 100 sheep from the territory of the Republic of Serbia (farms from Progar and Svilajnac) was tested. All sheep were of the Württemberg breed and clinically healthy, as determined by a veterinarian examination before blood sampling. Sheep blood samples were taken by puncture of v. jugularis in the amount of 3ml in vacutainers with EDTA as an anticoagulant and transported to the laboratory where they were stored in a refrigerator at 6°C until the moment of DNA isolation.

DNA extraction from blood

Invitrogen PureLink kit was used to isolate DNA from sheep blood. Genomic DNA was extracted from 50 µl of whole blood according to the manufacturer's instructions (Applied Biosystems, Thermo Fisher Scientific). DNA was resuspended in 50 µl or 100 µl of elution buffer. Extracted DNA samples were stored at 4°C until use.

Since the blood clotted in a certain number of samples, the isolation procedure was started by combining part of the clot (600µl) and PBS in tubes with ceramic beads. After addition, the tubes were centrifuged at 6,500 Rpm for 40 seconds at room temperature. Thereafter, the further isolation procedure was the same as mentioned above.

Table 1. Sets of primers and probes for the determination of sheep *prnp* alleles [11]

136 (A/V)	Primers	PrP-136F: 5'-GCCTTGGTGGCTACATGCT-3
		PrP-136R: 5'-CGGTCCTCATAGTCATTGCCAAAAT-3
	Probes	PrP-136-Ala-VIC: 5'-CTCATGGCACTTCC 3
		PrP-136-Val-FAM: 5'-CTCATGACACTTCC 3
154 (R/H)	Primers	PrP-154 F: 5'-TGGCAATGACTATGAGGACCG-3
		PrP-154 R: 5'-TGGTCTGTAGTACACTTGGTTGGG-3
	Probes	PrP-154-Arg-FAM:5'-ACTATCGTGAAAACAT-31
		PrP-154- His-VIC: 5'-TACTATCATGAAAACATG-3
171 (R/Q)	Primers	PrP-171F: 5'-ACCCCAACCAAGTGTACTACAGA-3
		PrP171R: 5'-GTCATGCACAAAGTTGTTCTGGT-3
	Probes	PrP-171-Gln-VIC: 5'-CCAGTGGATCAGTATAGT-3'
		PrP-171-Arg-FAM: 5'-CAGTGGATCGGTATAGT-3

Determination of codon 136, 154 and 171 polymorphisms (qPCR, TaqMan assay)

Polymorphism determination of codons 136, 154 and 171 was performed on a StepOne™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Oligonucleotide sequences and probes, for codon differentiation 136, 154 and 171 were designed by the commercial service (Assay by Design Service, Applied Biosystems) for *prnp* genes in sheep. Primers for each codon were marked: one with fluorescent dye (VIC) and the other with FAM. Tubes with a volume of 0.5 ml were prepared for each codon. In each tube were added: 2.7 μl of DNAse free water, 5 μl of polymerase (TaqMan Universal Master Mix, Applied Biosystems, Thermo Fisher Scientific), appropriate probes 1 and 2 - 0.2 μl and 0.45 μl of each primer (F, R). This mixture was transferred to a well on a PCR plate and 1 μl of DNA isolated was added. Quantitative PCR (qPCR) amplification was performed using the TaqMan method in a 10 μl reaction. Amplification was performed according to the manufacturer's instructions (Applied Biosystems).

RESULTS

PrP genotypes of sheep

The frequency of PrP genotypes tested on 100 sheep from the territory of the Republic of Serbia, based on codons 136, 154 and 171 is shown in Table 2.

Table 2. The frequency of PrP genotypes

PrP genotype	N	%
ARQ/ARQ	34	34
ARR/ARQ	29	29
ARR/ARR	6	6
AHQ/AHQ	10	10
ARQ/AHQ	18	18
VRQ/VRQ	1	1
ARQ/VRQ	2	2
Total	100	100

N – number of sheep

By analyzing the *prnp* gene polymorphisms, we found that two polymorphisms occur at each codon. Codon 136 contains alanine (A) and alanine/valine (A/V), codon 154 arginine (R) and arginine/histidine (R/H) and codon 171 glutamine (Q) and glutamine/arginine (Q/R) (Table 3).

The ARQ allele was the most prevalent, and the predominant genotype was ARQ/ARQ. Sheep with ARR/ARQ genotype form the second most represented group in this study. The VRQ/VRQ genotype is very rare and has been reported in only one case. Sheep with ARR/ARR genotype are considered the most genetically resistant, which was recorded in 6 cases.

Table 3. Polymorphisms of codons 136,154 and 171

	136	**154**	**171**
1	A	R	Q
2	A/V	R/H	Q/R

According to the National Scrapie Plan of Great Britain (NSP) [15], depending on the genotype, the sheep are divided into groups according to sensitivity, i.e. resistance to scrapie, which is shown in Table 4.

Table 4. Different levels of sensitivity base on genotypes of sheep

	N
NSP 1	6
NSP 2	28
NSP 3	55
NSP 4	/
NSP 5	3

NSP1 – Sheep that are genetically most resistant to scrapie, NSP2 - Sheep that are genetically resistant but need careful selection for further breeding, NSP3 - Sheep that are genetically poorly resistant to scrapie (requires very careful selection), NSP4 - Sheep that are genetically susceptible to scrapie and which should not be used for breeding, NSP5 - Sheep that are genetically most sensitive and which should not be used for breeding [15].

The most characterized polymorphisms occur at codons 136, 154, and 171, with genotype VRQ being the most susceptible and ARR the most resistant. The established genotype shows that the examined sheep mostly belong to the group that is not particularly genetically resistant to scrapie, so it is necessary to focus on selective breeding in order to minimize the risk of developing this disease.

DISCUSSION

Determination of *prnp* gene polymorphisms in sheep is performed worldwide, with more than 80 breeds of sheep in Europe, the Americas, and Asia examined [16]. According to data obtained from the sheep scrapie control program in the United Kingdom, all genotypes except the ARR/ARR genotype belong to groups of sheep that are susceptible to scrapie.

This is the first study regarding the examination of genetic makeup of sheep related to scrapie susceptibility conducted on a sheep population in the Republic of Serbia. Our results showed that only 6% of the examined sheep have the ARR/ARR genotype, which is related to genetic resistance to scrapie. The ARQ/ARQ genotype was present in 34% of the examined sheep, the ARR/ARQ genotype in 29%, the ARQ/AHQ genotype in 18%, and the AHQ/AHQ genotype in 10%, while the ARQ/VRQ and VRQ/VRQ genotypes were present in the smallest percentage (ARQ/VRQ - 1%, VRQ/VRQ - 2%).

The most frequent genotype was ARQ/ARQ and it is already known as the most common haplotype. Sheep with ARQ/ARQ genotype are susceptible to scrapie, but not all exposed sheep of this genotype get scrapie and if they do, usually have a long incubation period and prolonged survival [17]. Genotyping in the neighboring countries has also shown that ARQ is present with a high frequency: in Bosnia and Herzegovina it is present with 64.29% in Pramenka sheep breed [18], in Croatia it is present with 67.4% in the Istrian sheep breed [19], in the Hungarian breed Tsigai at 53.45% [20], etc.

Research conducted by Baylis et al. (2004) shows that the risk of developing scrapie in British breeds is highest in sheep with the VRQ/VRQ genotype. However, the frequency of this genotype is very rare. The ARR/ARR genotype was observed in 21% of sheep, the ARQ/ARQ genotype in 12% of sheep, the ARR/ARQ genotype in 28% of diseased sheep, the ARQ/AHQ genotype in 6% of sheep, and the AHQ/AHQ genotype in 1% of sheep [21].

According to Groschup et al. (2007) research performed on German breeds has shown the prevalence of ARR/ARR genotype was 35.4%, ARQ/ARQ genotype - 16.9%, ARR/ARQ genotype - 46.7%, ARR/VRQ genotype - 0,7% and an average of 0.1% in sheep with ARQ/VRQ, ARR/AHQ and ARQ/AHQ genotypes [9].

De Andrade et al. (2013) in their research analyzed blood of 3 Brazilian sheep breeds. In all analyzed breeds, the majority of the animals were AA homozygous for the 136 codon. The most frequent genotype for codon 154 was RR, and genotypes QQ and QR were the most frequent for codon 171 [22].

The established genotype shows that the examined sheep mostly belong to the group that is not particularly genetically resistant to scrapie, so it is necessary to focus on selective breeding in order to minimize the risk of developing this disease.

Although no case of scrapie has been registered in the last ten years, the obtained results indicate that the examined sheep population has genetically little resistance to classical scrapie. However, susceptibility to this disease should be considered individually for each breed, given that there are differences in the susceptibility of sheep with the same genotypes, so the authors plan to broaden the study in order to analyze all Serbian indigenous breeds of sheep. Sheep genotyping is very important to conduct in order to increase the level of genetic resistance to scrapie that would also

enable the reduction and eventually eradication of the scrapie. These measures would also indirectly protect the public health from the potential BSE cases.

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Authors' contributions

All authors carried out the molecular genetic studies. SN, SJ and IV drafted the manuscript. SN, SAK, MA and IV participated in the design of the study. All authors read adn approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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IDENTIFIKACIJA OPTIMIZOVANOM TAQMAN PROBOM QPCR METODOM GENA REZISTENCIJE NA SCRAPIE KOD OVACA NA TERITORIJI REPUBLIKE SRBIJE

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Ovčija svrabež je infektivno neurodegenerativno oboljenje iz grupe transmisivnih encefalopatija koje uzrokuje promene u moždanom tkivu ovaca i koza. Oboljenje je uzrokovano akumulacijom proteinaza-rezistentne izoforme prion proteina. Osetljivost ovaca na ovo oboljenje uslovljena je polimorfizmom PrP gena. Genetska otpornost na ovčiju svrabež prevashodno je uslovljena kodonima 136, 154 i 171. Najotpornije su ovce sa ARR genotipom, dok se ovce sa VRQ genotipom smatraju najosetljivijim. Mnoge zemlje su sprovele programe eradikacije ovčije svrabeži bazirane na korišćenju priplodnih ovnova koji imaju rezistentan genotip. Ovakav program još nije sproveden u Republici Srbiji. U cilju ispitivanja genetskog statusa ovaca u Srbiji u pogledu otpornosti na ovčiju svrabež, optimizovali smo *TaqMan* probu, kvantitativne lančane reakcije polimeraze (qPCR) tehnike za sva tri kodona. Ispitivanjem uzoroka krvi od 100 ovaca primenom RT-PCR ustanovljeno je da je većina ovaca AA homozigot za kodon 136. Za kodon 154 najčešći genotip je RR, a za kodon 171 genotip QQ.