Study of Frequency of Scrapie Resistant Genotypes in Țurcană Breed

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Abstract

Transmissible spongiform encephalopathies are caused by prions, which are pathological isoforms of normal proteins encoded by host organisms. The tests were performed on Țurcană breed sheep from a private farm in Sibiu County. Blood samples were taken from adult sheep and lambs born in 2021, and the next batch of samples was collected from 3-4-month-old lambs born in 2022. Blood samples were collected from the jugular vein, on EDTA, in a quantity of 6 ml/animal, and refrigerated at 4°C for transport to the laboratory. The analysis was performed in the molecular genetics laboratory of DSVSA Sibiu and at the National Reference Laboratory for Animal Health and Diagnosis in Bucharest. Sheep are classified into five classes of scrapie resistance: highly resistant, resistant, low resistance, susceptible, and highly susceptible. In 2021, resistant lambs accounted for half of the tested population. Among adults, 57.07% were resistant to the disease, 32.59% were in the low resistance group, and 10.34% were susceptible and highly susceptible. In 2022, 50.77% of lambs tested for scrapie were included into the resistant groups and 49.23% into the low resistance, susceptible, and highly susceptible groups. For breeding, only resistant sheep from the first two groups should be used and only in exceptional cases, sheep with low resistance from the third group. We recommend testing for scrapie resistance at the first sign of the disease, selecting sheep according to genotype, and purchasing rams that have been tested and found to be highly resistant for breeding purposes.

Keywords: small ruminants, spongiform encephalopathy

1. Introduction

Transmissible spongiform encephalopathies are caused by prions, which are pathological isoforms of a normal protein (prion protein or PrP) encoded by host organisms. Under certain conditions, this normal protein undergoes a post-translational modification of its secondary structure, acquiring new properties such as: exceptional resistance to physical and chemical factors, transmissibility similar to infectious agents [1].

The term "prion" (meaning "infectious protein") was introduced into molecular biology by Stanley Prusiner of the University of San Francisco in 1982 [2]. Prions (formerly known as protovirins, virinos, latent viruses) are a class of subviral infectious agents with a molecular weight of 27,000-30,000 D, unique in that they are resistant to all inactivation processes that degrade nucleic acids (heat, UV rays, ionizing radiation) [3]. Prions remain an enigma, a "perplexity" of molecular biology, because admitting their existence as infectious agents, lacking nucleic acid and composed exclusively of proteins, would argue for the overthrow of the central dogma of molecular biology, by accepting the coding and multiplication of this infectious prion protein, not through the nucleic acid in the structure of the prion agent itself, but through the nucleic acid of the host cell [4]. Prion proteins are normally present in all mammalian cells, participating in the structure of cell membranes.

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The incubation period is 2-5 years. The disease usually occurs in adult animals. There is no treatment or vaccine. It is not transmitted to humans.
The disease is usually transmitted from mother to foetus or through the placenta, faeces, bedding.
Clinical signs of the disease: behavioural disorders (nervousness, isolation); intense pruritus (scratching, loss of wool); tremors, ataxia; abnormal posture, stiff gait; progressive weight loss; slow progression, invariably fatal [5].
The environment remains contaminated for years.
In short, the approach has been one of surveillance, control, and eradication, not treatment. These include the following measures: culling of positive animals; movement restrictions; hygiene and disinfection; disposal of specified risk materials (brain, marrow); genetic selection programs (e.g., ARR-resistant alleles) [6,7].
The export of live sheep and goats is carried out from authorized sanitary-veterinary units, subject to sanitary-veterinary control [8].
Measures in line with the EU legislation and coordinated by ANSVSA have been implemented in Romania for transmissible spongiform encephalopathies (TSE) in sheep, particularly scrapie [9].

2. Materials and methods

Blood samples were collected from the jugular vein, on EDTA, in a quantity of 6 ml/animal and refrigerated at 4^0C for transport to the laboratory. The analysis was performed in the molecular genetics laboratory of the Veterinary and Food Safety Directorate in Sibiu and at the National Reference Laboratory for Animal Health and Diagnosis in Bucharest, part of the National Veterinary and Food Safety Authority.
The samples were genotyped at the PRNP locus using the primer extension technique, which involves the following steps [10.11]:

1. Blood sample collection with K3 EDTA;
2. DNA extraction from samples; determination of DNA template quality and quantity;
3. Pre-Snap Shot PCR reaction for DNA amplification;
4. Purification of PCR product on columns to remove NTP primers and inhibitors;

Genetic analysis to determine susceptibility to scrapie involves capillary electrophoresis and determination of polymorphism at the three loci, following the primer extension reaction.
This is performed in the presence of fluorescently labelled deoxyribonucleotides (ddNTP) on an ABI Prism 313 x l Applied Biosystem genetic analyser [12,13].
To analyse the results of electrophoretic profiles, the GeneMapper R software provided with the device or the PeakScanner program was used [14].
The PCR product is purified using the High Pure Purification Kit (Roche) method [15]:
- Adjust the PCR product to a final volume of 100 µL;
- add 500 µL binding buffer to the collection tubes;
- add 100 µL of amplification product to 500 ml of binding buffer in the collection tube.;
- transfer 600 µL of solution into a filter placed on a new collection tube;
- centrifuge at 1600 g for one minute;
- add 500 µL washing buffer over the filter;
- centrifuge at 1600 g for one minute.

Performing the primer extension reaction involves using a genetic analyser that determines the sequence of nitrogenous bases and detects SNP polymorphisms at the level of the three codons. This ABI-Prism 3130 x1 Genetic Analyzer (Applied Biosystem) is a high-performance genetic analyser (performing electrophoresis in 16 capillaries simultaneously) that can analyse 192 samples simultaneously, being an instrument used in most molecular genetics laboratories around the world [16.17].
To perform electrophoresis, a mixture of HI-DI formamide and molecular weight marker was prepared [18].
The primers used in the reaction are fluorescently labelled, with each nitrogenous base corresponding to a peak and a characteristic colour. During electrophoresis of the PCR product in capillaries filled with gel, the fluorescently labelled DNA passes in front of a laser beam with which the device is equipped. This excites each labelled molecule and converts the fluorescent values into the nucleotide sequence in the form of a matrix [19].
The polymorphism present at the level of the three codons (136, 154, and 171) is detected at the level of the electropherogram [20].
During the two years of study, samples were collected from a total of 319 adult sheep and 97 lambs.

In 2021, 319 adult sheep and 32 lambs were tested, and in 2022, tests were performed on 65 lambs remaining on the farm for breeding.

3. Results and discussion

In year 2021, both adult sheep and lambs retained for breeding were tested.

Based on the results obtained from the analysis of the frequency of genotypes presented in Table 1, it can be seen that lambs that were genetically highly resistant to scrapie (Group I) with the ARR/ARR genotypes represent only 12.5%.

In risk Group II, which indicates genetic resistance to scrapie but requires caution when used in selection programs, two out of three genotypes were identified, namely: one ARR/AHQ lamb, representing 3.125%, and 11 ARR/ARQ lambs with a high frequency of 34.375%. Overall, lambs with resistance to escape that can be retained on the farm for breeding account for 50% of the analysed cases.

Group III, which includes lambs with low genetic resistance to scrapie, identified four genotypes out of six, namely: AHQ/ARH with a frequency of 3.125%; AHQ/ARQ with a frequency of 9.375%; ARH/ARQ with a frequency of 3.125%; and ARQ/ARQ with a frequency of 25%. Compared to the total number of animals analysed in this sensitive group, but which can be used for breeding in exceptional cases, 40.625% of lambs fall into this category.

In risk Group IV, which includes lambs susceptible to scrapie, only the ARR/VRQ genotype was identified, in a small proportion of only 3.125%. Similarly, in risk Group V, which includes sheep that are highly susceptible to scrapie and must be castrated or slaughtered, two genotypes, AHQ/VRQ and ARQ/VRQ, were found in a small proportion of 6.25%.

The total number of lambs that must be eliminated following the analyses because they belong to risk groups IV and V with increased sensitivity to scrapie accounts for 9.375%.

Table 1. Frequency of genotypes for the PRNP locus in Turcana sheep in Sibiu County, year 2021

The intensity of risk			
Risk specification	**Genotypes**	**Number of lambs**	**The percentage %**
I. Genetically highly resistant to scrapie	ARR /ARR	4	12.5
II. Genetically resistant to scrapie but require attention for use in selection programs.	ARR / AHQ	1	3.125
	ARR / ARH	0	0
	ARR /ARQ	11	34.375
III. Genetic resistance to scrapie - animals used in selection schemes to be avoided	AHQ / AHQ	0	0
	AHQ /ARH	1	3.125
	AHQ /ARQ	3	9.375
	ARH /ARH	0	0
	ARH / ARQ	1	3.125
	ARQ /ARQ	8	25
IV. Susceptible to scrapie	ARR / VRQ	1	3.125
V. Very susceptible to scrapie and must be castrated or slaughtered	AHQ /VRQ	1	3.125
	ARH / VRQ	0	0
	ARQ / VRQ	1	3.125
	VRQ/VRQ	0	0

Genotypes consisting of all allele variants in the three codons were considered when examining genetic predisposition to scrapie. The homozygous ARR/ARR genotype appears to be the only one resistant to scrapie, with only one case of infection reported worldwide [21].

In a herd affected by the disease, the annual incidence is usually quite low (0.5–2%). In the absence of adequate prophylactic measures, there is a significant increase in incidence, which can reach 10% of the infected population, and the mortality rate is always 100% [22]. The study we conducted also recorded an incidence of 9.375% of sensitive genotypes without prophylactic measures being taken.

Due to the high incidence among lambs, it was decided that in the same year, 2021, the entire

adult herd, numbering 319 head, would be tested for scrapie resistance.

On the basis of the results obtained, Table 2 was drawn up, showing the proportion of sheep in the five risk classes. Of the total number of sheep tested, 44 sheep, representing 13.8%, fall into group I, with the ARR/ARR genotype, which is highly resistant to scrapie.

In group II, sheep genetically resistant to scrapie but which require attention for use in selection programs, the following genotypes were found: ARR/AHQ with a frequency of 1.57%, ARR/ARH with a frequency of 1.26%, and ARR/ARQ with a frequency of 40.44%. Total scrapie-resistant sheep falling within the first two risk groups: 57.07%.

Group III, comprising sheep with low genetic resistance to scrapie, which should be avoided in selection schemes but are acceptable in exceptional circumstances, includes the following genotypes: AHQ/ARQ with a frequency of 4.07%, ARH/ARH with a frequency of 0.31%, ARH/ARQ with a frequency of 1.88%, and ARQ/ARQ with a frequency of 26.33%. No sheep in this group were found to have the AHQ/AHQ and ARH/ARH genotypes. The total number of sheep falling into risk group 3 is 32.59%.

The total number of sheep in the first three risk groups is 57.07% + 32.59% = 89.66%, which is 0.96 percentage points less than the testing of lambs in the same year.

Risk group IV, which includes sheep vulnerable to scrapie with the ARR/VRQ genotype, accounts for 3.76% of sheep.

Risk group V, comprising sheep that are highly sensitive to scrapie, includes four genotypes, three of which were found: AHQ/VRQ with a frequency of 0.31%, ARQ/VRQ with a frequency of 5.96%, and VRQ/VRQ with a frequency of 0.31%. The ARH/VRQ genotype was not found. Total sheep in risk group V: 6.58%.

The sheep that must be removed from the herd and slaughtered belong to groups IV and V, representing 10.34%. These sheep and rams were removed from breeding in order to limit the incidence of scrapie in the herd.

Table 2. Frequency of genotypes for the PRNP locus in adult sheep of the Turcana breed in Sibiu County, year 2021

The intensity of risk			
Risk specification	**Genotypes**	**Number of lambs**	**The percentage %**
I. Genetically highly resistant to scrapie	ARR /ARR	44	13.8
II. Genetically resistant to scrapie but require attention for use in selection programs.	ARR / AHQ	5	1.57
	ARR / ARH	4	1.26
	ARR /ARQ	129	40.44
III. Genetic resistance to scrapie - animals used in selection schemes to be avoided	AHQ / AHQ	-	0
	AHQ /ARH	-	0
	AHQ /ARQ	13	4.07
	ARH /ARH	1	0.31
	ARH / ARQ	6	1.88
	ARQ /ARQ	84	26.33
IV. Susceptible to scrapie	ARR / VRQ	12	3.76
V. Very susceptible to scrapie and must be castrated or slaughtered	AHQ /VRQ	1	0.31
	ARH / VRQ	-	0
	ARQ / VRQ	19	5.96
	VRQ/VRQ	1	0.31

Since we received the test results at the end of 2021, we did not have enough time to take measures to remove adult sheep vulnerable to scrapie from breeding. So, we decided to test the lambs born in 2022, with the idea that in 2023 we would use tested young animals from our own flock to replace those that had been culled.

In 2022, 65 male and female lambs aged 3-4 months were tested. After collecting and analysing the samples, we obtained the

frequency of genotypes shown in Table 3. Based on the results, we determined the proportion of scrapie-resistant lambs that will be selected on the farm as future breeding animals. In this case, we were only interested in the resistant groups, the lambs in groups I and II.

The analysis of the data presented in Table 3 shows that lambs that are genetically highly resistant to scrapie (Group I) with the ARR/ARR genotype represent 13.85%.

In risk group II, which signifies genetic resistance to scrapie but requires caution for use in selection programs, two genotypes out of three were identified, namely: four ARR/AHQ lambs, representative of 6.15%, and 20 ARR/ARQ lambs with a high frequency of 30.77%. No lambs fell into the ARR/ARH genotype.

Out of all lambs with scrapie resistance that can be kept on the farm for breeding, they make up 50.77% of the cases analysed. Compared to 2021, there's a small increase of 0.77 percentage points. Without any action, the situation will get worse.

Group III, which includes lambs with low genetic resistance to scrapie, identified two genotypes out of six, namely: ARH/ARQ with a frequency of 3.07% and ARQ/ARQ with a frequency of 33.85%. The genotypes AHQ/AHQ, AHQ/ARH, AHQ/ARQ, and ARH/ARH were not found.

In all, 36.92% of lambs were tested for this sensitive group, but they can be used for breeding in exceptional cases.

In risk group IV, which includes lambs vulnerable to scrapie, no lambs with the ARR/VRQ genotype were identified.

Also, in risk group V, which includes sheep with high sensitivity to scrapie and which must be castrated or slaughtered, two genotypes, AHQ/VRQ and ARQ/VRQ, were identified in 12.31% of cases. The total number of lambs that must be eliminated following the analyses because they belong to groups IV and V with high sensitivity to scrapie is 8.

Table 3. Frequency of genotypes for the PRNP locus in Turcana sheep in Sibiu County, year 2022

The intensity of risk			
Risk specification	**Genotypes**	**Number of lambs**	**The percentage %**
I. Genetically highly resistant to scrapie	ARR /ARR	9	13,85
II. Genetically resistant to scrapie but require attention for use in selection programs.	ARR / AHQ	4	6,15
	ARR / ARH	-	0
	ARR /ARQ	20	30,77
III. Genetic resistance to scrapie - animals used in selection schemes to be avoided	AHQ / AHQ	-	0
	AHQ /ARH	-	0
	AHQ /ARQ	-	0
	ARH /ARH	-	0
	ARH / ARQ	2	3,07
	ARQ /ARQ	22	33,85
IV. Susceptible to scrapie	ARR / VRQ	-	0
V. Very susceptible to scrapie and must be castrated or slaughtered	AHQ /VRQ	1	1,54
	ARH / VRQ	-	0
	ARQ / VRQ	7	10,77
	VRQ/VRQ	-	0

The total frequency of genotypes is shown in Figure 1. From the analysis of this figure, it can be seen that the most common genotype in the sheep flock in the two years analysed is ARQ/ARQ in 144 cases out of 416 analysed, followed by the resistant ARR/ARR with 57 cases and ARR/ARQ with 31 cases.

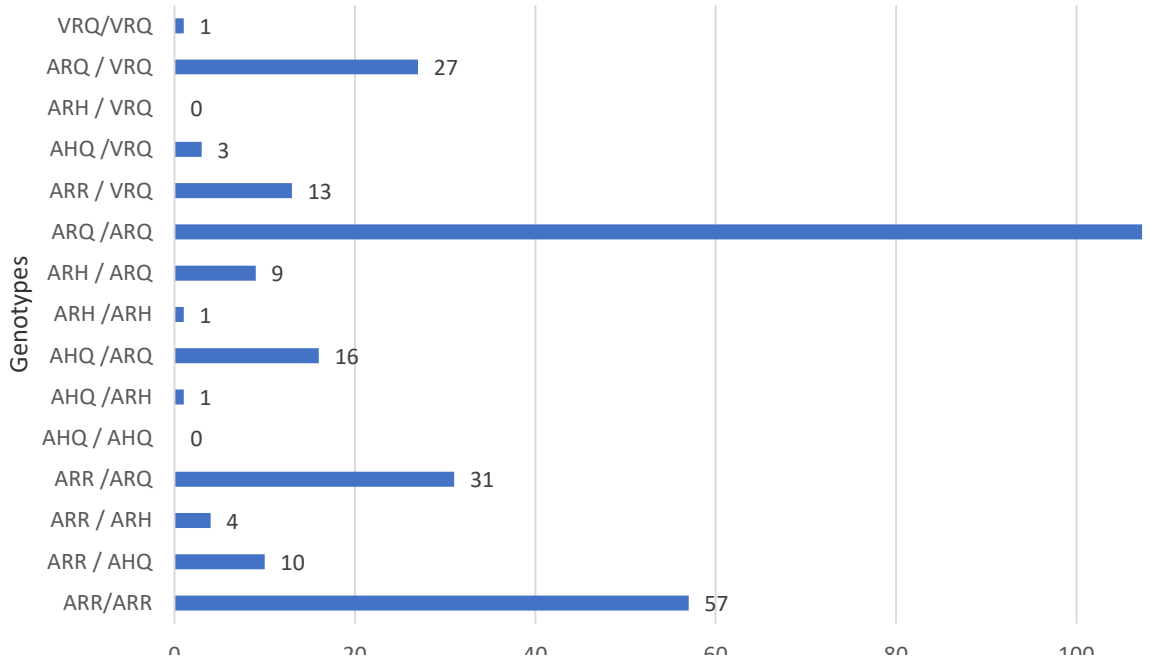

Figure 1. Total frequency of genotypes for the 2 years studied

Sampling and testing of adult sheep was carried out in September 2021, at the beginning of the mating season. As a result, it was not possible to eliminate scrapie vulnerability from groups IV and V of the breeding program, as they were already pregnant.

In this situation, measures to reduce the incidence of scrapie were to be taken in the summer of 2022, when, after weaning, both unproductive sheep and those with a genotype vulnerable to scrapie were to be slaughtered and replaced with young sheep tested in 2021, originating from genotypes resistant to scrapie.

In parallel with the culling of adult sheep vulnerable to the disease, measures were taken to purchase rams that are highly resistant to scrapie (group I) and to replace rams in the flock that belonged to genotypes III, IV, and V.

As a result of these measures, the incidence and manifestation of scrapie in lambs since 2023 has been sporadic and reduced to 1.5%.

Therefore, we did not proceed with harvesting and testing, which is very costly. Although the Romanian state has committed to subsidizing testing, there are often no funds available, and the costs are borne by the owner. To avoid being put in the situation we went through, we intend to test all lambs for scrapie resistance again next year.

We believe that in order to reduce the incidence of scrapie in sheep flocks, testing to select only scrapie-resistant genotypes for breeding is a method that must be applied regardless of whether or not the sheep are under official production control (OPC).

4. Conclusions

Testing for scrapie in lambs and adult sheep of the Țurcană breed on the farm during 2021-2022 allowed the following conclusions to be drawn:

Among the lambs tested in 2021, 50% were identified as resistant to scrapie, 40.625% as having low resistance, and 9.375% as having increased sensitivity.

Of the adult sheep tested in 2021, 57.07% were resistant, 32.59% were low resistant, and 10.34% were susceptible and highly susceptible to scrapie.

In 2022, 50.77% of lambs tested for scrapie fell into the resistant groups, 36.92% into the low resistance group, and 12.31% into the sensitive and highly sensitive groups.

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