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Comparison of the ovine prion protein genotype profiles of breeds Tigaie with Black Head and Merino

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Abstract: A comparison between a rare old breed and a common breed of ovine prion protein (PrP) genotype profiles was performed in 3 Romanian sheep flocks. The total number of animals used in the research was 698 sheep, of which 53 were Merino breed (MB) rams, 12 were Tigaie with Black Head breed (TBHB) rams, 340 were MB ewes, and 293 were TBHB ewes. The PrP genotype was investigated by identification of codons 136, 154, and 171 and by gene sequencing between codons 101 and 227. All sheep groups contained the ARR, AHQ, ARH, ARQ, and VRQ haplotypes, with frequencies ranging from 1.37% (ARH in TBHB ewes) to 51.71% (ARQ in TBHB ewes). TBHB had a low prevalence of AHQ (2.05% in ewes, 4.17% in rams), ARH (1.37% in ewes, 4.17% in rams), and VRQ (1.88% in ewes, 8.33% in rams). The highest haplotype in TBHB was ARQ (51.71% in ewes, 37.50% in rams). ARQ was the most frequent haplotype in the MB (49.41% in ewes, 42.45% in rams). MB had a low prevalence of AHQ (3.24% in ewes, 6.60% in rams), ARH (1.47% in ewes, 5.66% in rams), and VRQ (3.53% in ewes, 9.43% in rams). The results indicated that 66.66% of TBHB rams and 68.26% of TBHB ewes have scrapie-resistant genotypes, despite the absence of a genetic selection program for scrapie. The proportion of scrapie resistant alleles was higher in the TBHB rams than in the MB rams.

Key words: TSEs, PrP genotyping, scrapie

1. Introduction

Scrapie, a naturally occurring disease in sheep and goats, belongs to a group of neurodegenerative disorders referred to as transmissible spongiform encephalopathies (TSEs). Other TSEs include Creutzfeldt–Jakob disease; Gerstmann–Sträussler–Scheinker syndrome; kuru; transmissible mink encephalopathy; bovine spongiform encephalopathy; chronic wasting disease in elk, mule deer, and white-tailed deer; exotic ungulate encephalopathy; and feline spongiform encephalopathy (1).

Scrapie was the first TSE to be demonstrated to be transmissible (2). The exact nature of the scrapie agent is unknown (3), but it is thought that, like other TSEs, it is caused by misfolded protease resistant proteins called prions (4).

A critical aspect in the pathogenesis of TSEs is the host gene-encoded prion protein PrP. The primary transcript of PrP's open reading frame (ORF) comprises 256 amino acids, 209 of which are left after processing. Twenty-five of PrP's codons exhibit 32 amino acid substitutions. These gene mutations and polymorphisms are associated with the incidence of many TSEs (5–7).

In sheep, the amino acid codon numbers 136 (A/V), 154 (R/H), and 171 (Q/R/H/K) of the PrP gene have been linked to a significant effect on resistance to classical scrapie (8). These codons were investigated in several countries to develop sheep breeding programs (6, 9–12). Combinations of polymorphisms at these 3 codons give rise to 5 commonly seen haplotypes (alleles): ARR, AHQ, ARH, VRQ, and ARQ. These 5 alleles can be arranged to give 15 PrP diploid genotype combinations commonly found in sheep.

Using those genotypes, sheep susceptibility to scrapie was classified into 5 classes (13): class 1 (ARR/ARR genotype) is for sheep very resistant to scrapie; class 2 (ARR/AHQ, ARR/ARH, and ARR/ARQ genotypes) is

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for sheep that are genetically resistant to scrapie but need special attention for inclusion in selection programs; class 3 (ARQ/ARH, ARQ/AHQ, AHQ/AHQ, ARH/ARH, AHQ/ARH, and ARQ/ARQ genotypes) is for sheep with a lower genetic susceptibility to scrapie; class 4 (ARR/VRQ genotype) is for sheep that are sensitive to scrapie; and class 5 (AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ genotypes) is for sheep that are very sensitive to scrapie.

In our previous study, we described the PrP ORF haplotypes' frequency in a Romanian scrapie-infected farm. The prevalent haplotype identified was ARQ, and, consequently, the sheep with scrapie were predominantly ARQ (ARQ/ARQ = 81%, AHQ/ARQ = 9.5%). A very low frequency of the VRQ haplotype was described in scrapie-positive sheep (9.5%) (12).

Goldmann et al. (6) reported that a large number of the additional amino acid polymorphisms have been found to be associated with the ARQ haplotype, but their relation to scrapie is, in most cases, unknown. Only a few studies have suggested a partial resistance to natural scrapie: cysteine–arginine substitution at codon 151 in Icelandic sheep (14), threonine–methionine substitution at codon 112 in Japanese Suffolk and Corriedale sheep (15), and arginine–histidine substitution at codon 143 in natural goat scrapie (16).

White et al. (2010) cited in their background the studies of Melchior et al. (2010) and Hagenaars et al. (2010): "Scrapie eradication programs in the U.S. and Europe include a large contribution of breeding animals toward resistant genotypes, and classical scrapie prevalence is in decline" (17–19).

European Regulation (EC) No. 999/2001 states that "Member States which introduce breeding programmes shall submit regular reports to the Commission in order to enable the programmes to be scientifically evaluated, in particular with regard to their impact on the incidence of TSEs but also on genetic diversity and variability and on the maintenance of old or rare ovine breeds or of those that are well-adapted to a particular region. The scientific results and overall consequences of the breeding programmes shall be evaluated regularly, and where necessary, those programmes shall be amended accordingly." (20).

In the farm described by Otelea et al. (2011), a stamping-out procedure has been applied as part of the Romanian Scrapie Control Program, and unfortunately all old or rare breeds were eliminated (12).

The aim of this study was to investigate the frequencies of prion protein gene (PrP) alleles and genotypes within 3 sheep flocks in Romania of a rare ovine breed, Tigaie with Black Head (also named Carabasa), to compare the results with the same data obtained in Merino sheep. For genotyping, the entire sequence between codons 101 and 227 of the PrP gene was investigated by sequencing and melting curve analysis of codons 136, 154, and 171.

2. Materials and methods

The flocks selected for this study had at least 30 breeding ewes and had no suspected clinical cases of scrapie. The following sheep were used in the PrP genotype study: 53 Merino breed (MB) rams, 12 Tigaie with Black Head breed (TBHB) rams, 340 MB ewes, and 293 TBHB ewes.

Blood samples were taken from the jugular vein and processed according to standard procedures for determination of only the 3 codons 136, 154, and 171 of PrP.

The methods used for PrP genotyping have been previously described (12,20,21). Briefly, DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany); genotyping was performed with a LightCycler Scrapie Susceptibility Mutation Detection Kit (TIB MOLBIOL, Berlin, Germany); allelic variants were identified with Roche FastStart reagents LightCycler FastStart DNA Master^{PLUS}HybProbe (Roche, Mannheim, Germany); and the melting curve analysis was performed with a LightCycler 2.0 Real-Time PCR System (Roche, Germany).

The polymerase chain reaction (PCR) was set up in a volume of 20 µL with 5 µL of DNA solution and the following volume of reagents/reaction: 0.1 µL Ampli GoTaq Polymerase Promega (5 U/µL), 1.5 µL $MgCl_2$ (25 mM), 2 µL dNTP mix (10 mM), 2.5 µL buffer 10X for polymerase, 0.6 µM of forward primer(5'-GGTCAAGGTGGTAGCC ACAGTCAGTGGAAC-3'), and 0.6 µM of reverse primer (5'-ATCACCCAGTACCAGAGAGAATCCCAGGCT-3') (12,21). Amplification was performed at a temperature regime of: 95 °C for 10 min (denaturation); 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 60 s, for 40 cycles; and 72 °C for 10 min (12,21). The thermocycler used was an iCycler (BIO-RAD, Hercules, CA, USA). PCR products (5 µL) were analyzed on a 2% agarose gel (80 V for 45 min) containing ethidium bromide in TBE buffer (10 mM Tris, 2.75 g boric acid/L, 1 mM Na_2 EDTA). The visualization was performed in a UV transilluminator and the images were captured with a Polaroid camera and video documentation system (Bioprofil-Vilber Lourmat). The length of the amplified fragment was 402 bp.

The PCR reaction products were purified using a Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA), and then the concentration and purity of the products were evaluated by spectrophotometry (Eppendorf BioPhotometer, Hamburg, Germany).

The amplified products were sequenced by standard dideoxynucleotide analysis using a previously described protocol (20). In each reaction 6 ng of purified DNA and 2 pM of primer were used. The primers were the same as those used in the first amplification (12,21). The precipitation of the DNA sequencing product was performed using a BigDye XTerminator Purification Kit,

(Applied Biosystems, Foster City, CA, USA). Complete PrP haplotypes were defined by reading the entire sequence between codons 101 and 227.

The sequences were analyzed using BioEdit software and the CLUSTALW tool for multiple sequences alignment. Mutations were detected after comparison with a reference sequence (GenBank accession no. U67922). Sequences with novel mutations were sequenced in both directions on the DNA and manually verified.

3. Results

The frequencies of each class of scrapie susceptibility found in rams and ewes are illustrated in Figure 1. There was considerable variation in class distribution and generally a low variation between breeds. In both breeds, class 2 was the most frequent, followed by class 3 and class 1.

The group of TBHB rams did not contain genotypes of class 4. In the group of MB rams, class 2 was the most frequent, followed by class 3 and classes 1, 4, and 5. Moreover, in the group of TBHB rams, class 2 was the most frequent, followed by class 1 and classes 3 and 5. The group of TBHB ewes contained an unclassified genotype (ARQ/VRR).

The frequencies of the different haplotypes are shown in Table 1. All sheep groups contained the ARR, AHQ, ARH, ARQ, and VRQ haplotypes.

TBHB has a low prevalence of AHQ, ARH, and VRQ. The highest haplotype in TBHB was ARQ. ARQ was also the most frequent haplotype in MB. MB has a low prevalence of AHQ, ARH, and VRQ.

The frequency distribution of scrapie genotypes obtained in studied flocks is shown in Table 2. MB has the following genotypes: ARR/ARR, ARR/AHQ, ARR/ARH, ARR/ARQ, AHQ/ARQ, ARH/ARQ, ARQ/ARQ, ARR/VRQ, ARH/VRQ, ARQ/VRQ, and VRQ/VRQ. In addition to the genotypes identified in MB, TBHB has genotypes AHQ/AHQ, ARH/ARH, and ARQ/VRR.

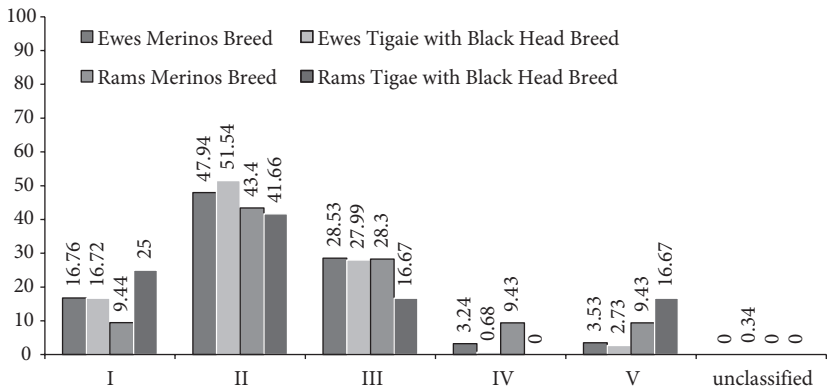

Figure 1. Frequency of scrapie susceptibility classes within the Merino and Tigaie with Black Head breeds in 3 scrapie-free farms from Romania.

Table 1. Frequency of PrP haplotypes within the Merino and Tigaie with Black Head breeds of 3 scrapie-free farms in Romania.

Haplotypes	Merino				Tigaie with Black Head			
	♂		♀		♂		♀	
	No.	%	No.	%	No.	%	No.	%
ARR	38	35.85	288	42.35	11	45.83	251	42.83
AHQ	7	6.60	22	3.24	1	4.17	12	2.05
ARH	6	5.66	10	1.47	1	4.17	8	1.37
ARQ	45	42.45	336	49.41	9	37.50	303	51.71
VRQ	10	9.43	24	3.53	2	8.33	11	1.88
VRR	0	0.00	0	0.00	0	0.00	1	0.17
Total	106	100	680	100	24	100	586	100

The sequencing analysis of the ovine prion protein gene (PRNP) fragment from p22556 to p22958 yielded similar results with those obtained from the melting curve analysis done by RT-PCR. In the amplified fragment (402bp) of PRNP, the polymorphic codons 136, 154, and 171 (Figure 2) were identified, confirming their commonness. In addition, an uncommon ARQ/VRR genotype was discovered (Figure 3).

4. Discussion

The current concept considers ARQ to be the wild-type form of the PrP allele, and the other alleles to be derived from it by single point mutations (22), and our results support that. ARR/ARR is the genotype associated with scrapie resistance, and a major goal of the Romanian scrapie program is to increase the proportion of homozygote rams carrying this allele.

Despite a higher number of scrapie-resistant ewes (with genotypes included in classes 1 and 2), the frequency of the ARR/ARR genotype (very resistant to scrapie) is still low and that can have a negative impact on the genetic selection program. Also, by using this small group of ARR homozygote ewes, the protection of genetic biodiversity in the national scrapie programs will be difficult. A modified selection program that will conserve all ARR heterozygote rams (but without the VRQ allele) can be considered.

Compared to the rams, the ewe groups had lower frequencies of genotypes with risk for scrapie. The highest number of ewes was in class 2, which classifies sheep as genetically resistant to scrapie but with "special attention" for use in selection programs. By genotyping the lambs resulting from the breeding effort to find out if they are ARR homozygous (if they originate from a mating with an ARR homozygous ram or ewe) or if they at least carry the

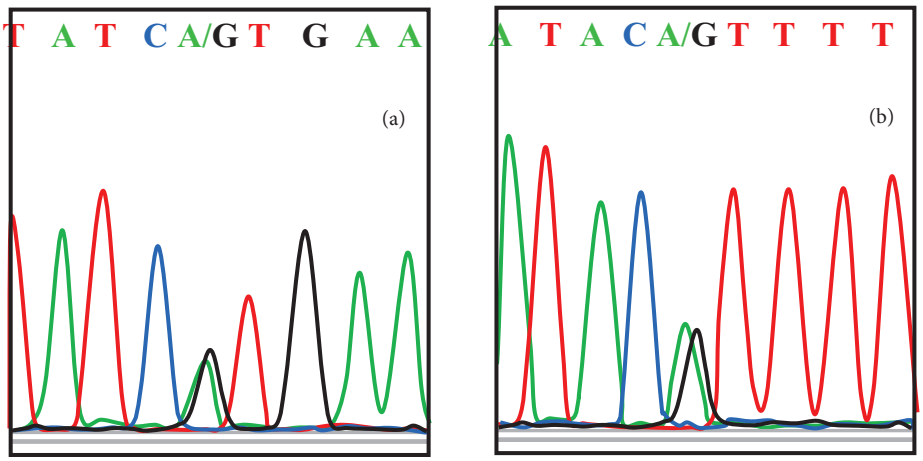

Figure 2. PrP gene polymorphisms: (a) Arg-His substitution in codon 154 (MB, female, 24 months, scrapie negative, genotype AHQ/ARQ); (b) His-Arg substitution in codon 143 (MB, female, 4 months, scrapie negative, genotype ARH/ARQ); (c) His-Gln substitution in codon 171 (MB, female, 24 months, scrapie negative, genotype ARH/ARQ); (d) Ile-Thr substitution in codon 185 (TBHB, female, 48 months, genotype ARR/ARQ); (e) His-Arg substitution in codon 143 (TBHB, female, 42 months, scrapie positive, genotype ARR/ARQ); (f) His-Gln substitution in codon 171 (TBHB, male, 36 months, genotype ARH/ARQ).

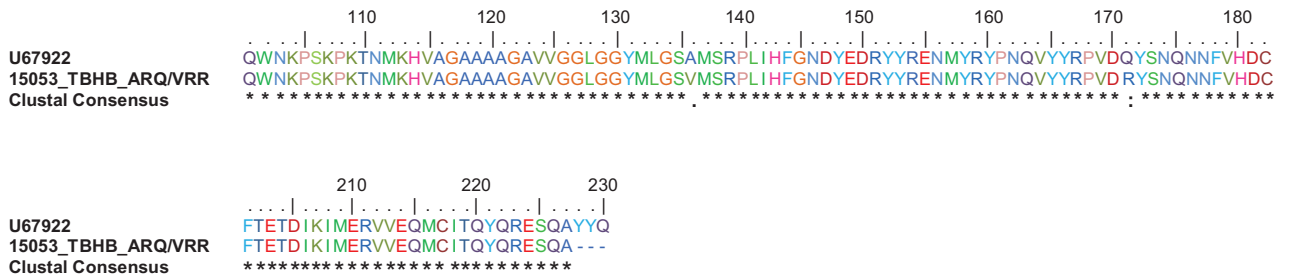

Figure 3. The ovine prion protein amino acid sequence of the ARQ/VRR genotype as shown in the BioEdit software with the CLUSTALW tool.

ARR haplotype (if they originate from a mating with a non-ARR homozygous mate), the decision can be made whether these lambs should be preferably used for breeding or for fattening. However, for flocks without scrapie selection programs, the proportion of resistant sheep (class 1 and class 2) was high. Usually, the selection of TBHB rams is based on mother milk productive performance. It is unclear whether the traditional selection of rams in TBHB plays an indirect role in this high proportion of scrapie-resistant genotypes or if this is just a random event.

The PrP gene polymorphism is also evidence for the genetic biodiversity of TBHB and MB. In this study, ovine genotyping revealed the presence of 6 PrP gene allelic variants in 2 different sheep breeds (Table 1), while Kutzer et al. (2002) identified 7 allelic variants of the same gene in 33 different German sheep breeds (21). Also, Kutzer et al. (2002) revealed distinct variations among PrP genotype frequencies in different breeds. The Texel breed showed over 17 genotype variants, while Friesian Milk sheep only had 7 different genotypes (23). In our study, TBHB showed a complex genotype distribution and 13 genotype variants (Table 2).

As in other studies (23,24), VRR was a rare haplotype. The VRR haplotype was found in TBHB with genotype ARQ/VRR, and in other studies it was in genotypes VRR/VRR, ARR/VRR, and VRR/VRQ (23). The scrapie susceptibility of the ARQ/VRR genotype is uncertain (6,24).

Statistically, the PrP genotype profiles of TBHB and MB generally had similar values. Only the proportion of scrapie-resistant alleles was higher in the TBHB rams compared to the MB rams. The genotyping results corroborate the increased biodiversity of the TBHB genome and a high proportion of scrapie-resistant sheep in this breed, despite the absence of a scientific program of scrapie breeding selection.

Table 2. Frequency distribution of scrapie genotypes within the Merino and Tigaie with Black Head breeds of 3 scrapie-free farms in Romania.

Genotype	Merino				Tigaie with Black Head			
	♂		♀		♂		♀	
	No.	%	No.	%	No.	%	No.	%
ARR/ARR	5	9.43	57	16.76	3	25.00	49	16.72
ARR/AHQ	3	5.66	12	3.53	1	8.33	1	0.34
ARR/ARH	3	5.66	7	2.06	1	8.33	0	0.00
ARR/ARQ	17	32.08	144	42.35	3	25.00	150	51.19
AHQ/AHQ	0	0.00	0	0.00	0	0.00	5	1.71
AHQ/ARH	0	0.00	0	0.00	0	0.00	0	0.00
AHQ/ARQ	4	7.55	10	2.94	0	0.00	1	0.34
ARH/ARH	0	0.00	0	0.00	0	0.00	1	0.34
ARH/ARQ	2	3.77	3	0.88	0	0.00	5	1.71
ARQ/ARQ	9	16.98	84	24.71	2	16.67	70	23.89
ARR/VRQ	5	9.43	11	3.24	0	0.00	2	0.68
AHQ/VRQ	0	0.00	0	0.00	0	0.00	0	0.00
ARH/VRQ	1	1.89	0	0.00	0	0.00	1	0.34
ARQ/VRQ	4	7.55	11	3.24	2	16.67	6	2.05
VRQ/VRQ	0	0.00	1	0.29	0	0.00	1	0.34
ARQ/VRR	0	0.00	0	0.00	0	0.00	1	0.34
Total	53	100.00	340.00	100.00	12.00	100.00	293.00	100.00

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