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The genetic resistance of rams from Turcana breed to Ovine Transmissible Spongiform Encephalopathy (scrapie)

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Abstract

Since 2005, the European Union Member States have been compelled to utilize programs that foster resistance to ovine transmissible spongiform encephalopathy (TSE) as a part of the measures to eradicate TSE at the national level (2001R0999—EN; Annex I to Commission Decision 2002/1003/EC). In sheep, some polymorphisms of the gene PRN-P (gene of PRioN Protein) have a major influence on sensitivity/resistance to TSE. As concerns the classical scrapie, the polymorphisms of the gene PRN-P from codons 136 (A or V), 154 (R or H) and 171 (R, Q or H) strongly influence ovine sensitivity to prion infections. The present paper shares the results of genetic structure analysis of the PRN-P gene locus for three representative ecotypes of the Romanian Turcana breed. 245 Turcana rams, the ecotypes of Hateg, Bala de Bistrita and Sibian, were genotyped at the PRN-P locus by means of primer extension analysis, and the final products were analysed on a Genetic Analyzer ABI Prism 3130 xl. The frequency of genes by haplotypes were identified for each ecotype, and the individuals were categorised in risk groups based on the frequency of the identified genotype, with a view to applying assisted selection at the molecular level.

Key words: TSE, scrapie, *PRN-P* gene, polymorphism, prion, Turcana breed

Introduction

Prions are the transmissible pathogens that cause a class of neurodegenerative diseases in mammals, including Creutzfeld-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in sheep. Cattle with BSE have been implicated as the cause of one human transmissible spongiphorm encephalopathy (TSE), variant CJD, through the consumption of beef from affected animals [18, 3]. Naturally occurring prion diseases may be acquired, inherited, or from sporadic origins (i.e., unknown environmental or genetic cause). Thus, cross-species transmission of TSE may extend across subfamilies and super orders. Although there is no evidence of sheep scrapie transmission to humans in more than 250 years of exposure [17], uncertainties associated with species barriers have prompted many countries to develop policies to eliminate all TSE-affected animals from their food chains, including scrapie in sheep.

Scrapie - is a naturally occurring neurodegenerative disease of sheep. The disease is experimentally transmissible to cattle, goats and laboratory animals via oral, parenteral and intracerebral routes using homogenates of brain or lymphoid tissues from infected animals [13]. The mode of transmission from ewe to lamb or between adults under field conditions is not known. However, oral exposure to fetal membranes or to pastures grazed by infected animals has been implicated as a possible route of vertical and horizontal transmission [14]. Susceptibility to ovine scrapie is controlled by a combination of host genetics. Polymorphisms at residues 136, 154 and 171 are associated with susceptibility to both, experimental and natural scrapie [22]. Experimental transmission of BSE to sheep revealed

that animals carrying the genotype ARR are as well resistant to BSE when administered via the oral route. This finding is of great importance considering the risk that BSE could reach, via contaminated feed, small ruminants and could thus circulate — unrecognized and confused with scrapie — within European sheep and goat populations [11].

Prionic protein (PrP) - During the course of prion disease, a largely protease-resistant aggregated form of PrP, designated PrP^{Sc}, accumulates mainly in brain, and may be the main or only constituent of the prion [15]. The posttranslational conversion of the cellular isoform of prion protein (PrP^{C}) into the scrapie isoform of prion protein (PrP^{Sc}) is the fundamental process underlying both the transmission and pathogenesis of the prion disease [16]. In contrast to pathogens carrying a nucleic acid genome, prions appear to encipher strain-specific properties in the tertiary structure of PrP^{Sc}. Transgenic studies argue that PrP^{Sc} acts as a template upon which PrP^{C} is refolded into a nascent PrP^{Sc} molecule through a process facilitated by another protein [26]. Because no differences in primary sequence were found between PrP^{C} and PrP^{Sc} [20], the two species are believed to differ only in their conformation. After PrP^{C} is synthesized in the endoplasmic reticulum, it transits through the Golgi to the cell surface where it is bound by a glycophosphatidyl inositol (GPI)-anchor (5, 6). At or near the cell surface, PrP^{C} is either metabolized or converted into PrP^{Sc} [7]. PrP^{C} seems to re-enter cells through caveolae-like domains (CLDs), a subcellular compartment defined biochemically by membranes rich in cholesterol and glycosphingolipids; this compartment also contains many GPI-anchored proteins [16].

Prion gene polymorphism (*PRN-P*) - With over 30 SNPs already reported, the ovine prion gene (PrP) shows an unusually high level of genetic variation [31]. Increased resistance to classical scrapie is associated with a prion protein gene (*PRN-P*) haplotype allele encoding one alanine (A) and two arginine (R) at codon positions respectively 136, 154, and 171 (i.e. ARR). Conversely, a haplotype encoding valine (V), R, and glutamine (Q) at those positions (i.e. VRQ) is associated with increased susceptibility or attack rate [21, 23]. Haplotype alleles encoding three other forms of PrP (ARQ, AHQ, and ARH, where H is histidine) have intermediate associations with classical scrapie disease progression following exposure to the transmissible agent [32].

There were thus several genetic variants associated to the same level of resistance/ susceptibility to scrapie. Table 1 includes the wild type and mutant variants at the level of the three codons of interest, in positions 136, 154 and 171 (where there are two possible mutations).

Table 1. Allelic variants (wild type and mutant) in codons 136, 154 and 171 of *PRN-P* gene

Codon no.	Wild type		Mutant	
	Codon	Aminoacid	Codon	Aminoacid
136	G<u>C</u>C	A	G<u>T</u>C	V
154	C<u>G</u>T	R	C<u>A</u>T	H
171A	C<u>A</u>G	Q	C<u>G</u>G	R
171B[a)]	C<u>A</u>G	Q	C<u>A</u>T	H

Underling nucleotide represents the SNP mutation in the specific codon
a) These nucleotides are detected on the complementary DNA strand.

Genetic testing for the five most common haplotype alleles (i.e., ARR, ARQ, AHQ, ARH, and VRQ) is a key feature of scrapie eradication programs. Management decisions depend on which of the 15 possible combinations of these paired *PRN-P* haplotypes (i.e., diplotypes) are present in an animal [19]. Firstly, sheep that show resistance to classical scrapie based on their genotypes at codons 136, 154 and 171 are susceptible to atypical variants [2, 29]. Thus, selection for resistance to typical scrapie agents could put sheep populations at risk to infections by atypical variants such as Nor98, for example [27], the susceptibility to which is affected by a polymorphism at codon 141 [28]. In terms of PrP genetics, one classification of the GB scrapie cases examined in this study would place animals carrying any homozygous or heterozygous combination of ARR, AHQ or $AF^{141}RQ$ alleles, or any one of these alleles when paired with ARQ, as being susceptible to atypical scrapie infection, and animals heterozygous or homozygous for VRQ or homozygous for ARQ as being susceptible to classical scrapie disease [9]. Subsequently, studies on sheep susceptible/resistant to classical scrapie and experimentally infected with BSE infectious agent revealed that in most cases, resistance was associated with *PRN-P* variant M112T polymorphism (alleles TARQ) [8].

Biological material – Given that males have a high level of gene diffusion in the population, 245 Turcana rams were selected for genetic analysis concerning resistance/ susceptibility to natural scrapie, in order to find the resistant genotypes and include them in the selection schemes. Based on data provided by the National Agency for Improvement and Reproduction in Animal Technology, in Romania the breed structure is heterogeneous, being made up of 22 breeds and a group of metis, autochthonous breeds and imported breeds. Out of the total number of 255,551 sheep registered in the official system of production control, 242,854 sheep, i.e. 95 %, are of the Romanian breeds of Turcana, Tigaie, Merinos de Palas, Transylvanian Merinos, Merinos de Cluj and Romanian Caracul, while the rest of 12,967 individuals is represented by imported breeds [1]. Ţurcana is still the breed that holds the biggest proportion - about 73 % of the total number. There are four varieties within the breed: white, black, frosty and Ratca. The white variety is the most widespread. The more valuable ecotypes within this variety include: Turcana sibiana, Bala de Bistrita and Bala de Hateg [24], which were included in the present study.

Materials and Methods

The primer extension assay, a rapid and accurate method for the detection of sheep PrP genotype at codons 136, 154 and 171, based on the SNaPshot chemistry (Applied Biosystems, Foster City, CA, USA), which represents an adaptation of the primer extension technique, was used in present work. It includes two main steps: the first one is represented by the amplification of the target DNA sequence containing the polymorphic sites of interest. The second one is the primer extension reaction carried out with the SNaPshot® Multiplex System. This reaction consists of a single nucleotide extension of primers complementary to the target DNA and adjacent to the polymorphic sites of interest. During this step, the primers incorporate fluorescently labelled dideoxynucleotides (ddNTP), which are present in the SNaPshot chemistry. Primers are designed with tails of varying lengths at the 5'-end allowing their identification by size differences through a single electrophoresis in a fluorescence-based DNA sequencer [11]. The assay is carried out using a single tube reaction for each sample and the final reaction volume is minimised to reduce the amount of reagents.

DNA samples - The DNA from 200 µl fresh blood, collected in K-EDTA tubes, was extracted using *MagnaPure LC DNA Isolation Kit* (Roche) following the steps of the *DNA I Blood_Cells_High_Performance* protocol [12]. The extractions were performed on the MagnaPure Lc Automatic Nucleic Acid Extraction Robot (Roche) following the

manufacture's instructions. The blood samples were collected from rams registered in the official control of production, while the structure of the ecotypes is as follows: 123 individuals of the Hateg ecotype, 51 of the Sibian ecotype and 71 Bala de Bistrita ecotype.

The quality and quantity of DNA was spectrophotometrically detected using NanoDrop ND 1000 instrument (Thermo Scientific), the values situated between 1.7 and 2.0, and 50.8 and 283 ng/µl, respectively.

Template amplification – This stage allows the PCR amplification of a genomic DNA fragment that contains the studied polymorphisms. Each set of samples contains an additional positive and a negative control template (with water). The positive control contains 2 µl SnaPshot Multiplex control template, whose polymorphism is known.

PCR amplification was carried out in 25 µl final volume containing: 2 µl of DNA, 10x PCR buffer (100 mM Tris-HCl (pH 9.0), 15 mM MgCl2, 500 mM KCl - Pharmacia), dNTPs (200 µM each), 2.5 µl Primer Mix Snapshot, 1.5 IU Taq polymerase (Amersham Pharmacia). We amplified partial *PRN-P* coding region (GenBank accession number AJ223072 from nucleotide 287 to 613) with the primers described previously [11]. The amplification was performed in a DNA Thermal Cycler (Mastercycler Eppendorf) with a heat step of 3 minutes at 95^{o}C and 35 cycles of 60 seconds at 95^{o}C, 30 seconds at 57^{o}C and 30 seconds at 72^{o}C. The final extension step was realized at 72^{o}C for 10 minutes.

PCR products purification was achieved with *High Pure PCR Product Purification Kit* (Roche) according to the instruction provided with kit.

Primer extension reaction – The primer extension reactions were carried out in 7 µl reaction mixture containing 5 µl of the SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), 1 µl of pooled Snapshot Primers and 1 µl distilled water. 3 µl of the previously purified PCR product were added in each 200 µl PCR tube. The reaction was performed in a DNA Thermal Cycler (Mastercycler Eppendorf) through 25 cycles of 10 seconds at 96^{o}C, 5 seconds at 58^{o}C and 30 seconds at 60^{o}C.

Post Snapshot treatment – 1 µl of *Calf Intestinal Phosphatase* (CIP) was added in each reaction tube, and then incubated at 37^{o}C for 10 minutes and 15 minutes at 75^{o}C.

Capillary electrophoresis – A common reaction mixture was prepared containing for each sample 11.5 µl HI-DI formamide and 0.5 µl Gene Scan-120 LIZ size standard (Applied Biosystems), which was distributed in the electrophoresis plates. In each tube we transferred 1 µl SnaPshot treated with CIP. The plates were centrifuged at 2000g for 1 minute, then denaturated through the incubation of the electrophoresis plates at 95^{o}C for 5 minutes and 3 minutes at 4^{o}C. Labelled primers were visualised by electrophoresis with the ABI Prism ® 3130 xl Genetic Analyser (Applied Biosystems). The electrophoretic patterns were finally analysed by the Genemapper ® software (Applied Biosystems).

Results and discussion

The determination of the allelic phase has been inferred from *PRN-P* genotypes on the basis of the five *PRN-P* alleles described in the European sheep populations. Figure 1 presents the electrophoregrams obtained for three of the genotypes recorded at the *PRN-P* locus.

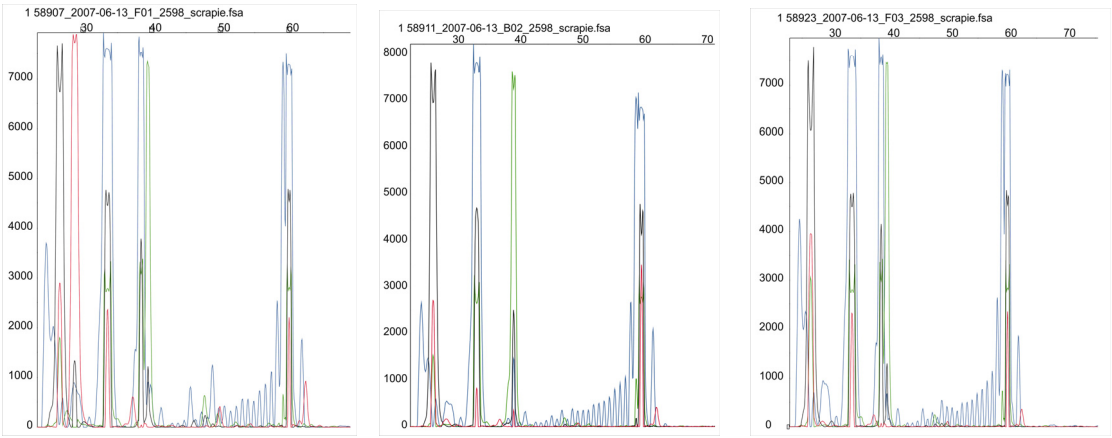

Fig. 1. Electrophoregrams with ARR/VRQ, ARQ/ARQ, and ARR/ARQ genotypes at *PRN-P* locus

***PRN-P* haplotype and genotype frequencies:** The frequency of the susceptible *PRN-P* haplotypes varies between different sheep breeds but is usually at a level that makes it impossible to remove these haplotypes in a single generation. Furthermore, strong selection for scrapie resistance would also result in a loss of genetic variability, especially in smaller breeds and can have affected any traits (by pleiotropy or by linkage) associated with *PRN-P* genotypes. This implies that selection against susceptible haplotypes should be implemented using breed specific selection strategies in order to be cost-effective and maintain genetic variation. In our populations the haplotype and genotype frequencies were determined for each ecotype, the data being recorded in tables 2 and 3.

Table 2. Frequency (%) of alleles by haplotypes in the three ecotypes of the Turcana breed

Turcana breed					
Hateg ecotype		Bala de Bistrita ecotype		Sibian ecotype	
Allele	Haplotype frequencies [30]	Allele	Haplotype frequencies	Allele	Haplotype frequencies
ARR	34.15	ARR	38.03	ARR	45.1
ARQ	53.67	ARQ	53.76	ARQ	41.18
AHQ	1.22	AHQ	5.63	AHQ	3.92
ARH	1.22	ARH	-	ARH	-
VRQ	8.94	VRQ	2.82	VRQ	8,82
VHQ	0.4	VHQ	-	VHQ	0.98
VRH	0.4				
N= 123	100	N= 71	100	N=51	100

ARQ and secondly ARR were the most common haplotypes in all three ecotypes. The most susceptible haplotype, VRQ, appears to have a moderate frequency (8.94% in Hateg ecotype and 8.82% in Sibian ecotype) compared with other breeds and therefore must be eradicated. Although in the Sibian ecotype there were no case of scrapie, in the herds of the Hateg ecotype sick animals were found (unpublished data), and the legal measures were taken by the competent authorities. The frequencies on haplotypes in the Turcana breed vary between 34.15 and 45.1 for the allele ARR, between 41.18 and 53.76 for the allele ARQ and between 2.82 and 8.94 for the allele VRQ. These frequencies are intermediary as compared to those recorded by other authors in different European breeds [10, 25].

The possible combination of the 5 amino acids encoded by the 3 different codons determines 15 possible genotypes at the *PRN-P* locus. Evidences have been accumulated indicating that the ARR allele is associated with a minimal susceptibility, particularly in case of the homozygous genotype ARR/ARR, while ARQ and VRQ confer the highest susceptibility, an intermediate effect being attributed to the other common alleles [21, 22, 23]. Great Britain, which is the country most affected by this disease, groups the genotypes in five major classes of risk within the National Scrapie Plan [4]:

-ARR/ARR : very resistant to scrapie

-ARR/AHQ, ARR/ARH, ARR/ARQ : resistant to scrapie

- ARQ/ARQ, ARQ/AHQ, ARQ/ARH, ,AHQ/AHQ, ARH/ARH, AHQ/ARH : little resistance to scrapie

-ARR/VRQ : susceptible to scrapie

-AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ : highly susceptible to scrapie

Taking into account these groups of risk, and the susceptibility / resistance to the disease, we have proceeded to group the genotypes in the three ecotypes in risk categories. The data concerning the frequency of genotype and the risk group are presented in Table 2.

Table 2. Frequency (%) of genotypes and their grouping in risk categories for the three investigated ecotypes

Group	Genotype	Hateg ecotype	Bala de Bistrita ecotype	Sibian ecotype
GROUP I	ARR/ARR	14.64	15.49	35.29
GROUP II	ARR/AHQ	0.81	9.86	
	ARR/ARH	-		
	ARR/ARQ	32.52	33.8	15.69
GROUP III	ARQ/ARH	2.44		
	ARQ/ARQ	28.46	33.80	23.53
	ARQ/AHQ	1.63	1.41	3.92
	ARH/ARH			
	AHQ/AHQ			1.96
	AHQ/ARH			
GROUP IV	ARR/VRQ	5.69	1.41	3.92
GROUP V	AHQ/VRQ			
	ARH/VRQ			
	ARQ/VRQ	12.19	4.23	13.73
	VRQ/VRQ			

Note: The genotypes ARQ/VRH (Hateg ecotype) and ARQ/VHQ (Hateg and Sibian ecotypes), containing two rare alleles (VRH and VHQ) were removed from the table.

Concerning the frequency of genotypes, the recorded data show a frequency between 14.64% and 35.29% of homozygotes ARR/ARR which will be used preferentially in the selection schemes. Other genotypes that have at least one ARR allele also occur with a considerable frequency (ARR/ARQ) between 15.69% and 33.8%, while the Bala de Bistrita ecotype records in addition a considerable percentage of genotypes ARR/AHQ. However, the proportion of homozygotes ARQ/ARQ is high, recording values of 33.8% in the Bala de Bistrita ecotype and somewhat less in the Hateg ecotype (28.46%) and the Sibian ecotype (23.53%).

Conclusions

One of the principal means of eradicating scrapie is through the use of selective breeding, which exploits the association between polymorphisms in the prion protein (PrP) gene and the risk of scrapie. Such breeding programmes aim to control scrapie by increasing the frequency of the *PRN-P* allele associated with the lowest risk of clinical disease (ARR) while reducing the frequency of the allele associated with the highest risk (VRQ). From April 2005, member states of the European Union were required to implement a compulsory breeding programme for resistance to TSE in sheep [5]. Consequently, a new long-term strategy for the NSP (National Scrapie Plan) was required that took into account this legislation and, in particular, the requirement that it be compulsory in flocks of 'high genetic merit'. Under the EU minimum requirements (strategy 1), animals carrying the VRQ allele must not be used for breeding within the flock and tested rams that carry the VRQ allele must not be sold to other flocks [5, 6]. Based on the number of replacement rams required for use within a flock, we estimated that genotyping 10% of rams bred would be more than adequate to cover the replacement rams for use within a participating flock and, hence, comply with this minimum requirement. The proportion of genotypes with a VRQ allele in the ecotypes Hateg, Bistriţa and Sibian were of 17.88%, 5.64% and 17.65% respectively, but in all the ecotypes of the Turcana breed there were over 10% homozygote ARR/ARR rams.

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