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GENETIC AND PATHOLOGICAL ASPECTS OF PRION PROTEIN (*PrP*) IN SHEEP BELONGING TO BOTOSANI KARAKUL BREED

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Abstract

By Real-Time PCR technique, the existence of polymorphism at the level of PrP gene (associated with susceptibility to scrapie) has been revealed in Botosani Karakul sheep. Practically, the polymorphism was analyzed at codons 136, 154 and 171. Among the five alleles (ARR, ARQ, AHQ, ARH and VRQ) incriminated in association with this disease in ovine species, only three of them were found in the Botosani Karakul breed determining the phenotypic expression of all six possible genotypes. The most common allele is ARQ (56.10%); the incidence of allele ARR (38.21%) is considerable, and the allele ARH has a low prevalence (5.69%). The most frequent genotype is ARQ/ARQ (47.97%), followed by genotype ARR/ARR (26.83%); the genotypes ARR/ARQ (14.63%) and ARR/ARH (8.13%) record moderate or relatively low frequencies, the other two genotypes (ARO/ARH = 1.63% and ARH/ARH = 0.81%) being met rarely in the population. The distributions of genotypes at the PrP locus make the total homozygosity (75.61%) to be well represented compared to the total heterozygosity (24.39%). In the Botosani Karakul breed, major discrepancies were observed between the empirical frequencies and those estimated, so that we witness a very significant genetic disequilibrium Hardy-Weinberg at the PrP locus. In the Botosani Karakul sheep, the prion genotypes that are associated in the highest degree with scrapie are completely absent (classes of risk R4 and R5). Thus, all individuals are resistant to scrapie (50%) (classes of risk R1) or have a low risk of contracting the disease (50%) (class of risk R3). This association represents a notable selective advantage of Botosani Karakul sheep compared to all other autochthonous or foreign sheep breeds.

Keywords*: Botosani Karakul sheep, prion, scrapie, TSEs.*

INTRODUCTION

One of the most fascinating areas of biomedical research is represented by the study of non-conventional transmission agents (NCTA) (Petit and Boucraut-Baralon, 1992). With this term there were designated infectious agents, still of controversial nature, responsible for degenerative diseases of the central nervous system: *acute transmissible spongiform encephalopathies* (TSEs). These diseases, always dramatic, encountered in humans and animals, possess a number of common features. TSEs are clinically characterized by a very long incubation period (from few months to several years - even decades) and a slow and antipyretic evolution (ataxia, tremor, abnormal posture), being inevitably fatal. The typical lesions are located in the gray matter of the central nervous system and consist of a vacuolar degeneration and sponginess of the neurons. From the epidemiological point of view, these diseases evolve sporadically, sometimes epizootic; they are transmissible, but have a pronounced hereditary component (Petit and Boucraut-Baralon, 1992; Hunter, 1999; Somerville, 2002). Such an entity with the role of transmissible infectious agent was named *prion* being composed of a protein with misfolded structure. This is the central idea of the *Prion Hypothesis* which remains in debate. Structurally, this particle can be in contrast to all other known infectious agents (viruses, bacteria, fungus, parasites) which contain obligatorily nucleic acids (either DNA, RNA, or both). Concept of *prion*, introduced in 1982 by Stanley B. Prusiner,

derives from the words protein and infection (short for *proteinaceous infectious particle)*, building thus his theory according to which a deviant form of a harmless protein could be an infectious agent, a transmitter of disease (Prusiner, 1982; 1991; 1998); otherwise, Prusiner was awarded the Nobel Prize in Physiology or Medicine in 1997 for his research on prions. The malady is transmissible to humans and other animal species, bearing different names (Hunter, 1999; Belay, 1999). In sheep, *prion* is the agent of scrapie disease. *Scrapie* is still known as prion disease of small ruminants, because sheep, goats and mouflons are species susceptible to scrapie (Beringue and Andreoletti, 2014).

Due to its connotations pathogenetic, scrapie is considered as an integrated system of intensive and profound studies with biomedical, economic and environmental approaches. The studies of molecular genetics relating to the prion protein took a large scale lately in sheep breeds in Western Europe, especially with the momentum of practicing the industrial crossbreeding for creation of new sheep types with high productive performance to satisfy the increasing demands of the growing market with productions of this species, in particular with the meat one (Fediaevsky et al., 2008). In Romania these studies are fairly recent history and took into account the Merino, Tsigai and Tsurcana breeds specialized mainly for meat or milk productions (Coşier, 2008; Otelea et al., 2011). The Botosani Karakul breed, specialized for the lamb pelt production, has benefited from a singular (almost episodic) approach in this regard, occasioned by implementation of a project of the Research and Development Station for Sheep and Goat Breeding Popauti in collaboration with the University of Bucharest (Kevorkian et al., 2011).

That is why this paper has like main objective the molecular-genetic characterization at the *PrP locus* of Botosani Karakul breed, strictly from the elite farm of this station, where sheep are reared in pure breed, to evaluate the incidence of sheep genotypes sensitive to TSEs and implicitly susceptible to developing prion disease in order to eliminate them from the flock.

MATERIALS AND METHODS

Genotyping of Botosani Karakul sheep at the PrP locus occurred in a random population of 123 animals reared in pure breed within the elite farm of the *Research and Development Station for Sheep and Goat Breeding Popauti* where the correct application of the selection criteria for the improvement of this breed leaves no doubt. We make this statement because, in its existence area, the purity of Botosani Karakul breed might be altered by some cross-breeding actions practiced by private shepherds, fact that can be reflected also on the genetic structure of this breed, including at the PrP locus level.

In order to identify some genes susceptible to contracting the scrapie disease, *biological samples were collected from the brainstem level* from sheep by classical surgical method (Kevorkian, 2010).

Extraction and isolation of genomic DNA from brain homogenate was performed using the kit "High Pure PCR Template Preparation" (Roche, 1999), respecting the manufacturer specifications (Kevorkian, 2010; Kevorkian et al, 2011).

The analysis of PrP locus was performed by Real-Time PCR technique at codons 136, 154 and 171. For this purpose there were used simultaneously the kits "LightCycler FastStart DNA Hybridization Probe MasterPLUS" (Roche Applied Science) and "LightCycler Scrapie Susceptibility Mutation" (TIB MOLBIOL) in accordance with the methodological rules of manufacturers (Kevorkian, 2010; Kevorkian et al., 2011).

Real-Time PCR reaction was carried out on Light Cycler 2.0 apparatus (Roche, 1999) and browsed the following steps: pre-incubation 95°C, 8 minutes, amplification with 45 cycles, melting curve and cooling to 40°C for 30 seconds. Each amplification cycle consisted of three stages: 95°C, for 10 seconds, 60°C, for 10 seconds and 72°C, for 15 seconds. Melting curve was also performed during three stages: 95°C, for 2 minutes, 45°C, for 1 minute and a step of gradually increasing of the temperature of each 0,2°C, from 45°C to 75°C, for 1 minute (Kevorkian, 2010; Kevorkian et al, 2011).

The gene and genotypic frequencies were calculated at the PrP locus. Also, we calculated the ratio between and homozygosity and heterozygosity for the prion protein locus. The *Hi square test* (χ^2) was used for comparison of the observed frequencies to the expected ones. Also there were assessed *the levels of risk regarding the contamination with scrapie* of

Botosani Karakul breed in accordance with the international standards established by the Monitoring Programme of TSEs adopted by the Council of Europe (2013).

RESULTS AND DISCUSSIONS

There are more than 15 known alleles of the PrP gene, but only five are used to characterize sheep at the PrP locus and therefore to assess their resistance / susceptibility level to scrapie. These alleles can be sorted by increasing order of susceptibility to scrapie, as follows: ARR, AHQ, ARH, ARQ and VRQ (Cosier, 2008; Otelea et al., 2011).

The purpose of this analysis was to identify, in the Botosani Karakul breed, possible polymorphisms of the gene coding for prion protein at codons 136, 154 and 171 in sheep incriminated to be associated with susceptibility to scrapie (Otelea et al., 2011).

Genetic structure

The polymorphism of PrP gene in tht Botosani Karakul sheep was revealed by detecting three alleles at this locus: ARR, ARQ and ARH. The distribution of these alleles is uneven. The ARQ allele (56.10%) is the most frequently encountered. Also, the allele ARR shows a considerable frequency (37.40%). Allele ARH has a low spreading (5.69%) (Figure 1).

The three alleles determine the occurrence of all six possible genotypes: three homozygous (ARR/ARR, ARQ/ARQ and ARH/ARH) and three heterozygous (ARR/ARQ, ARR/ARH and ARQ/ARH). The genotypes for PrP gene have a very disproportionate spreading. Almost half of the individuals are homozygotes of type ARQ/ARQ (47.97%), and a quarter of them are homozygotes of type ARR/ARR (26.83%), while the homozygotes for allele ARH are sporadically met in population (0.81%). Among heterozygotes, the individuals of type ARR/ARQ are the most widespread, but their incidence (14.63%) is moderate. The frequency of heterozygotes ARR/ARH is relatively low (8.13%), whereas the heterozygotes ARQ/ARH are very poorly spread (1.63%).

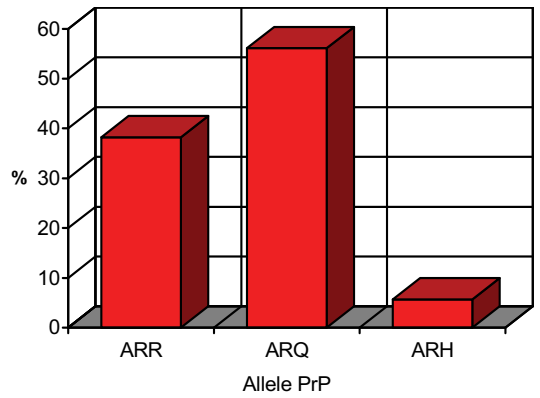
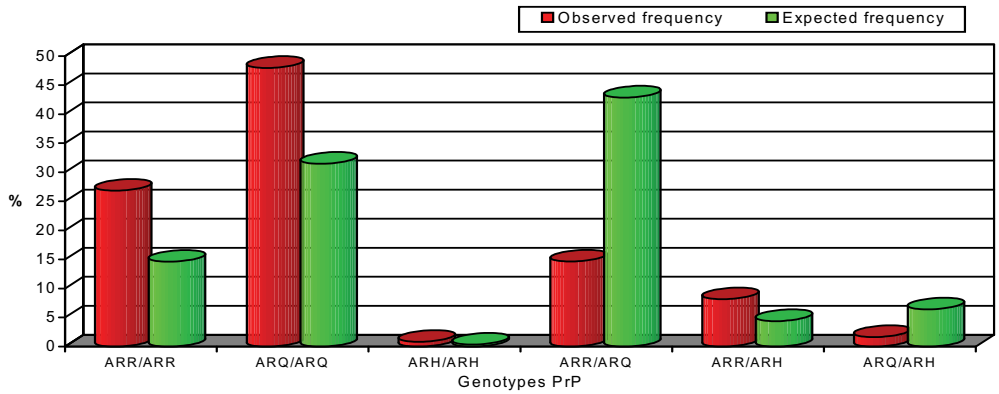


Figure 1. Allelic structure at the PrP gene locus in the Botosani Karakul breed

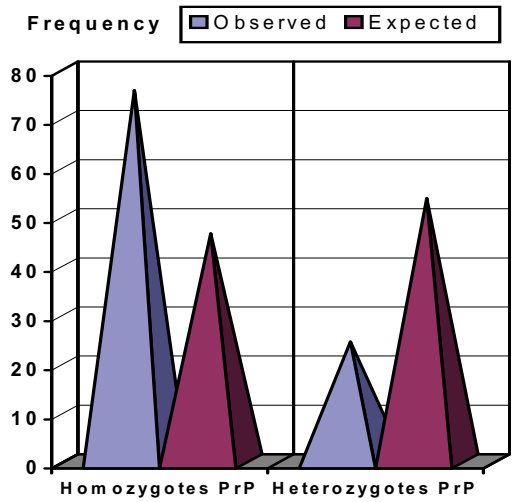
The large discrepancies were found between the observed and expected distributions. Thus, the homozygotes ARR/ARR and ARQ/ARQ occur more frequently than expected, but the greatest discrepancy is observed at the level of heterozygotes ARR/ARQ where their phenotyping is much lower than expectations of their occurrence. For this reason, the test χ^2 records a high value (44.9958***), exceeding even the most critical significance level (0.001). As such, the population is very significantly genetic unbalanced at the PrP locus (Figure 2).



χ^2= 44.9958***; L.D.=4; p<0.001

Figure 2. Genotypic structure at the PrP gene locus in the Botosani Karakul breed

In terms of general zygosity, the total number of homozygotes (75.61%) is three times higher than that of heterozygotes (24.39%). But because the estimation shows that the two categories of individuals would have occur in almost similar proportions (46.39% / 53.61%), there are great differences between practical and theoretical distributions, resulting a high value of the test χ^2 (34.3313***), the differences being, statistically, very significant. Therefore, also with regard the relationship between the total homozygosity and total heterozygosity, the population deviates very significantly from Hardy-Weinberg law of genetic equilibrium (Figure 3).



χ^2= 34.3313***; G.L.=1; p<0.001

Figure 3. Zygosity status at the PrP gene locus in the Botosani Karakul breed

Association of the PrP locus with scrapie disease in sheep

The process that triggers the disease is represented by the conversion of a normal protein, sensitive to the proteases, synthesized naturally in the brain of all mammals (PrP^c), into a mutant, abnormal structure, resistant to proteolysis, (PrP^{Sc}), specific marker of spongiform encephalopathies. This change occurs only after the prion protein was anchored to the cell membrane (Bossers, 1999; Priola et al., 2003).

In the Botosani Karakul breed (Table 1), the genotype ARQ/ARQ was recorded in 47.97% of animals. This genotype is associated with a relatively increased risk of developing the scrapie disease. The animals that possess genotypes ARQ/ARH and ARH/ARH present also some risks to develop the malady, but their frequency is low (1.63%) or very low (0.81%) in population. The three genotypes are included in the third risk class of developing the disease; it is considered that these individuals have a little resistance to scrapie and this fact requires attention at the time of mating. Some authors consider that these genotypes represent a low risk in the tested animal, but the risk is real in his descendant depending on the genotype of the other parent.

The animals with genotypes ARR/ARQ (14.63%) and ARR/ARH (8.13%) belong to the second class of risk. The individuals included in this class has a low development level of the disease, but depending on the genotype of partners with which they will be mated, their offspring could present different signs of disease development. However, it is considered that the risk is extremely low, both in the tested animal and in its progeny (descendant).

The animals with genotype ARR/ARR (26.83%) are included in the first class of risk (very low risk both in the tested animals and in their offspring) being highly resistant to the disease, being very recommended in the selection works of sheep.

Very important for the Botosani Karakul sheep, reared in pure breed, in comparison to other indigenous or imported breeds, is that the genotypes belonging to the risk classes 4 and 5 are missing; the individuals with these genotypes (containing allele VRQ both in homozygous status, but also in association with all the other alleles) are highly susceptible to scrapie, having a low resistance to the disease. For these reasons, the Botosani Karakul breed has an advantage from the perspective of genetic prophylaxis to scrapie. Thus, in this breed, thanks to more limited polymorphism at the PrP locus, all individuals have resistance to scrapie (50%) or low risk of contracting the disease (50%). In all the other breeds (which are older than Karakul, both local and foreign breeds) or hybrid entities (cross-breeds) of sheep, especially in those created by industrial cross-breeding, in which there is a more emphasized polymorphism at the PrP locus, the probability of scrapie occurrence is higher. Therefore, the prion polymorphism in ovine species, besides its pathological importance, might have phylogenetic connotations, too.

Table 1. Frequencies of PrP genotypes and their classification into risk groups for scrapie in Botosani Karakul breed (*according to the Department for Environment, Food and Rural Affairs - 2007*)

Risk class	Resistance level	PrP Genotype	Botosani Karakul sheep (%)	
Class 1 (R1)	The most resistant type to scrapie	ARR/ARR	26.83	
Class 2 (R2)	Genetically, resistant types to scrapie, but need a certain attention to be used in mating schemes	ARR/ARQ	14.63	22.76
		ARR/AHQ	-	
		ARR/ARH	8.13	
Class 3 (R3)	Less resistant types to scrapie and need a particular attention to be used in mating schemes	ARQ/ARH	1.63	50.41
		ARQ/AHQ	-	
		AHQ/AHQ	-	
		ARH/ARH	0.81	
		AHQ/ARH	-	
		ARQ/ARQ	47.97	
Class 4 (R4)	Genetically, susceptible sheep to scrapie and should not be used in mating, with the exception of a controlled breeding program	ARR/VRQ	-	
Class 5 (R5)	Very susceptible types to scrapie and should not be used at all in mating	AHQ/VRQ	-	-
		ARH/VRQ	-	
		ARQ/VRQ	-	
		VRQ/VRQ	-	

The limitation of prion polymorphism would be due, to a large extent, to natural selection exerted over time. However, not unimportant, it would be the artificial selection pressure to strengthen the production traits, including by moderate inbreeding for creating the breeding lines, as happens in the farm elite with Botosani Karakul sheep of our station. This assumption has occurred as a consequence of the results of investigations carried out (unpublished yet) by the National Reference Laboratory for Molecular Biology of the Institute for Diagnosis and Animal Health which revealed other prion genotypes too associated with the risk classes 4 and 5 in sheep of Botosani Karakul breed, but from private farms where the selection does not carried out following the most rigorous rules. However, these assumptions must be confirmed by further studies in an integrated context regarding the accuracy of selection and reproduction process.

Genotyping of the Botosani Karakul sheep for the PrP locus creates real prerequisites for implementing a selection program regarding the resistance to scrapie on whole area of the breed that must be applied in each sheep breeding scheme.

CONCLUSIONS

By Real-Time PCR technique, a population of Botosani Karakul sheep was genotyped at the PrP locus (associated with susceptibility to scrapie), being revealed a restricted polymorphism at its level.

Prion polymorphism of this breed is determined by the existence of three alleles, ARR (having a considerable incidence), ARQ (the most common) and ARH (with a low spreading), all three alleles contributing to the phenotypic expression of all six possible genotypes.

The genotypic panel is dominated by the genotype of ARQ/ARQ followed by genotype ARR/ARR; the genotypes ARR/ARQ and ARR/ARH record moderate or relatively low frequencies and the other two genotypes (ARO/ARH and ARH/ARH) are seldom met in population; at the PrP locus the homozygosity is three times more consistent than the heterozygosity.

The Botosani Karakul breed is in very significant genetic disequilibrium Hardy-Weinberg at the PrP locus.

In Botosani Karakul breed the PrP genotypes which are associated with scrapie in the highest degree are completely missing (risk classes 4 and 5); all individuals have resistance to scrapie (50%) (risk classes 1 and 2) or present low risk of contracting the disease (50%) (risk class 3).

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