Selection Role on Configuration of Resistance or Susceptibility Degree to Scrapie and on Genetic Diversity at *PrP* Locus in the Botosani Karakul Sheep Breed

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Abstract
The molecular-genetic profiles from the determinant locus of scrapie have been described in the Botosani Karakul breed. Sheep belonged to two farm types: elite farm from *Research and Development Station for Sheep and Goat Breeding, Popauti-Botosani* and several private production farms. The two farm types differ between them by selective specificities; in the farm elite the selection pressure is stronger and the selection criteria applied to sheep have been more accuracy than in the production farms. Sheep genotyping at the PrP locus was achieved by the Real-Time PCR method. By framing the individuals in risk classes concerning the prion disease, there were identified significant differences between the two farm types in terms of resistance or susceptibility to scrapie of sheep. The elite farm population is significantly advantaged as regards the molecular-genetic endowment at the PrP locus and low probability converting of the PrP^c normal protein in the PrP^{Sc} pathogenic isomorph compared to the population of production farms. The reasons for the different associations of prion genotypes with morbid phenomenon intensity in the two farm types must be sought exclusively in the characteristics of selection systems applied to the animals in each farm type. From the standpoint of informational statistics, there is a high degree of genetic similarity at the PrP locus between the two populations. Contrary to expectations, the genetic diversity of prion structures is more developed in the elite farm than in the production farms. The knowing importance of prion profiles was revealed in pursuit of genetic and veterinary prophylaxis of the sheep populations.

Keywords: genetic diversity, prion, Real-Time PCR, scrapie, sheep

1. Introduction

The prion protein isomorph is at the origin of neurodegenerative diseases generically called transmissible spongiform encephalopathies (TSEs), deadly diseases both for humans and animals [1, 2]. Casuistically and statistically, in most cases the manifestation field of this disease group is the ruminants, especially the farm ones, having recognized names for each species; bovine spongiform encephalopathy (BSE) or "mad cow disease" in cattle and scrapie in sheep, goats and mouflon [3]. Generically, scrapie is referred to the prion disease of small ruminants [4]. Among the ruminants from spontaneous fauna the disease can be found in deer as "chronic wasting disease" (CWD) and in several species of African antelope called "exotic ungulate encephalopathy" (EUE). Apart from herbivores, the disease is also manifested in felines - „feline spongiform encephalopathy” (FSE) and in minks - „transmissible mink encephalopathy” (TME) [5]. Genes coding for PrP or PrP-like protein were also detected in birds, reptiles, amphibians and fish [5-9]. In humans, the most common prion diseases

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are Creutzfeld-Jacob malady (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI), Kuru malady and Alpers syndrome (seen only in children) [3, 10].

The infectious component is certainly known to all these diseases: the prion agent or the infectious amyloid [11]. The prions are unconventional infectious agents of proteic nature, devoid of any nucleic acid [12]. Unlike other conventional types of microbes (viruses, bacteria etc.), the prions have not DNA or RNA, whereof it was thought to be necessary for any infectious agent in order to multiply within the host cell [13]. All these diseases present extracellular accumulation of amyloid consisting of the prion protein (PrP^c) in the pathological version (PrP^{Sc}). In sick individuals the normal variant (PrP^c) is in small amount. Although most of the proteins within a cell are easily cleaved, the prions resist to enzymatic cleavage which explains the rapid propagation of the disease. Also, the vehicle of this disease is not destroyed by any of the conventional sterilization methods [1, 2, 14]. Humans can be contaminated with the scrapie agent through the food chain consuming infected meat [15]. The illness is favored by the immune system dysfunction and it can be propagated from the periphery to the CNS and vice versa. The medical personnel accidentally can get sick [10].

The process that triggers the prion diseases is represented by the conversion of a normal protein, synthesized naturally in all mammalian neurons (PrP^c), into a mutant, abnormal one (PrP^{Sc}). Depending on the factor that induces the conversion of PrP^c into PrP^{Sc}, the nature of these disorders may be genetic (in the case of a point mutation of the PrP gene), infectious (as a result of consumption of contaminated food, the use of non-sterile surgical instruments, injection of hormones derived from cadaver pituitary) or sporadic (due to spontaneous mutations in the PrP gene, which increase the conversion rate of the prion protein, as well as to some unknown factors) [1, 2].

In ovine species, the determinant element in the occurrence of scrapie disease, with a gradual spectrum of morbid manifestation, is represented by the five alleles from the PrP locus level, the sequence presented being in relation to the increasing order of susceptibility to scrapie of animals: ARR, AHQ, ARH, ARQ and VRQ [16].

In farm animals, in addition to the reasons mentioned above, the predisposition to such diseases or their dissemination in populations are related to the technological factors of animal breeding and especially to the selection ones [17, 18]. For this reason, our paper has set as objective evaluating the resistance or susceptibility degree to scrapie in the Botosani Karakul sheep bred in different farm types in relation to the accuracy of selection criteria applied in the improvement process of this breed. That is all the more so this study conforms to the requirements of European Community policies on the eradication of transmissible spongiform encephalopathy in livestock and to the concept of security and safety food [15, 19].

2. Materials and methods

The investigations of molecular genetics were carried out on Botosani Karakul sheep populations bred in two farm types: elite farm and production (commercial) farms. Elite farm is located in the *Research and Development Station for Sheep and Goat Breeding, Popauti-Botosani*. The animals from the production farms belong to various private farmers whose livestock holdings are spread in the most representative influence area of Popauti Station, respectively in Botosani County. In the elite farm 178 animals were genotyped and in 27 production farms 2822 individuals were available for genotyping. It must be noted that in the elite farm the technological breeding systems were better, the selection criteria had a higher accuracy and the selective pressure phenomenon was more pronounced than in the production farms.

The biological samples for laboratory analysis consisted of venous sheep blood sampled in tubes containing anticoagulant (K_3EDTA).

The molecular genotyping of Botosani Karakul sheep was achieved at the PrP locus that is responsible for the occurrence of scrapie in small ruminants.

The extraction of genomic DNA from blood samples were performed using High Pure PCR Template Preparation Kit [20].

Genotyping of sheep at the PrP gene locus was achieved by the Real-Time PCR technique, that is based on the fluorometric detection principle, using the LightCycler Scrapie Susceptibility Detection Kit (TIB MOLBIOL) and LightCycler FastStart DNA Master Hybridization Probes [20]. A sense primer (forward) and an anti-sense primer (reverse) were used to amplify the DNA extracted, the operation comprising more amplification cycles of different temperature gradients. The amplicons were separated by agarose gel electrophoresis (2%) and visualized with UV trans-illuminator. The prion genotypes have been identified depending on the configuration and peak number of the melting curves. The confirmation of genotypes identified by Real-Time PCR technique was achieved by the sequencing technique; the obtained sequences were aligned with the reference sequence from the GeneBank database using the Clustal W application of the BioEdit software [20, 21].

The prion genotype frequencies were calculated for their framing into risk classes concerning the susceptibility degree to scrapie according to the *Department for Environment, Food and Rural Affairs* [22]. The values of allele frequencies from the PrP locus were necessary to estimate the genetic distances (D) by Nei method, to calculate the informational correlation coefficient ($R_{x,y}$) between the populations by Onicescu-Stefanescu method and to quantify the prionic genetic diversity (d) within the animal populations by Onicescu-Ștefanescu method [23]. The evaluation of genetic equilibrium in animal populations in the two farms types was achieved using the Hi^2 test (χ^2).

3. Results and discussion

By the Real-Time PCR technique the polymorphism gene coding for the prion protein has been identified at the level of 136, 154 and 171 codons in sheep belonging to the Botosani Karakul breed within the two farm types.

The detection of genotypes from the PrP locus level was possible analysing the peculiarities of melting curves.

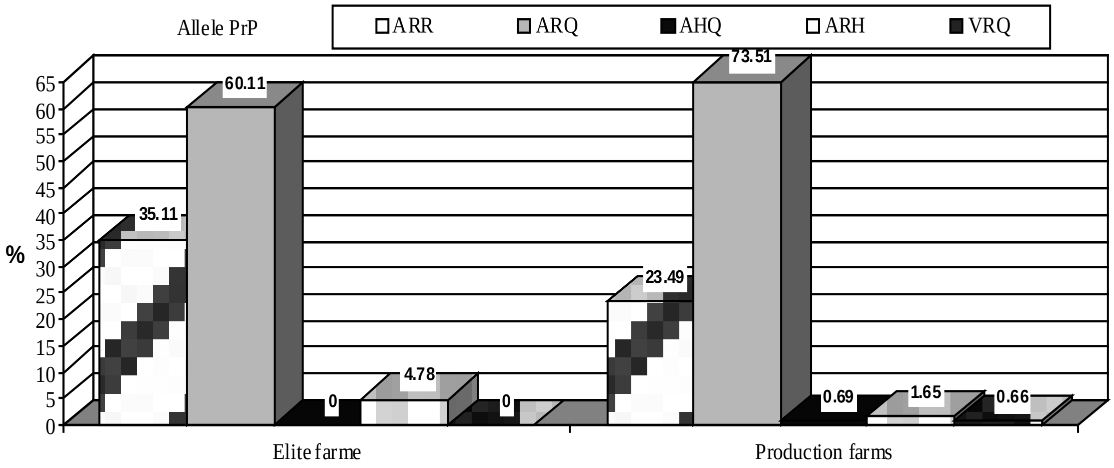
A previous genetic analysis at the PrP locus in the Botosani Karakul sheep revealed the characteristics of prion panel in this breed. The common note of populations of the two farm types is the abundance of ARQ/ARQ genotype which occupies almost half of the prion panel. Also the ARR/ARQ heterotype records frequencies to be taken into consideration. The incidence of the ARR/ARR homozygous type is moderate. The heterozygotes ARR/ARH and ARQ/ARH have a low spreading and the other genotypes are found sporadically in populations. Of the 15 possible genotypes in the Botosani Karakul sheep only 11 were expressed, the genotypes AHQ/ARH, AHQ/VRQ, ARH/VRQ and VRQ/VRQ missing from the prion panel. This genotypic configuration is due to the very significant presence of ARQ allele and to the moderate incidence of ARR allele which enters in reciprocal combinations both of homozygous type (ARR/ARR, but mainly ARQ/ARQ) and of heterozygous type (ARR/ARQ). The other three alleles (AHQ, ARH and VRQ) have a poor representation [24].

The differences on selection criteria, selection pressure and improvement technology applied in the two farm types are reflected in the configuration of prion panels in the two sheep populations of Botosani Karakul breed.

In sheep from elite farm only three alleles were detected (ARR, ARQ and ARH), while in sheep from production farms all five alleles prion were found. The ARR allele that is associated with the highest resistance to scrapie is more frequent by almost 12% in elite farm population (35.11%) than in the one of production farms (23.49%). Also, although the spread of ARH allele is weak in both populations, this allele that is associated with resistance to scrapie rather than with morbid phenomenon, it has an incidence of almost three times higher in the elite farm (4.78%) than in the commercial farms (1.65%). Instead, the ARQ allele associated with a high degree of contracting this disease is very common in both populations, but its incidence is higher by about 15% in the production farms (73.51%) than in the elite farm (60.11%). In addition, in production farms the alleles AHQ (which gives a good resistance to scrapie) and VRQ (which is associated to the highest degree with prionic morbid phenomenon) are also found, but their frequencies are extremely low (each one with about 0.7%) (Figure 1).

The number of alleles and their frequencies determine a corresponding expression of prion genotypes in the two farm types. In sheep of production farms, the presence of all five alleles determines the expression of 11 prion genotypes of the 15 possible: homozygotes for allele ARR, ARQ, AHQ and ARH and heterozygotes ARR/ARQ, ARR/AHQ, ARR/ARH, ARQ/ARH, ARQ/AHQ, ARR/VRQ and ARQ/VRQ. The heterozygotes AHQ/ARH, AHQ/VRQ, ARH/VRQ and homozygotes VRQ/VRQ are absent from the prion panel of these populations. In the elite farm the three alleles are found in all six possible combinations: homozygotes for allele ARR, ARH and ARQ and heterozygotes ARR/ARQ, ARR/ARH and ARQ/ARH (Table 1).

Another aspect of differentiation between the two farm types is represented by the genetic equilibrium status at PrP locus. In the elite farm the differences between the expression of prion genotypes and their appearance expectation are significantly large; on the contrary, in the production farms, the low value of χ^2 test shows that the population complies with Hardy-Weinberg law (Figure 1, Table 1).



χ^2 25.3874***; L.D.=7; p<0.001 0.9895; G.L.=13; p>0.05

Figure 1. Allele frequencies at the PrP locus in Botosani Karakul sheep depending on the farm type

The different expression of PrP alleles in prion genotype combinations occurs also on different resistance or susceptibility degree to scrapie of animals from the two types of livestock holdings.

In fact, the main objective of this study is revealing the framing in the five risk classes regarding the contamination of individuals within the elite and production farms with prionic agent [22] (Table 1).

Table 1. Frequencies of PrP genotypes and their framing into risk classes to scrapie in Botosani Karakul sheep depending on the farm type (*according to the Department for Environment, Food and Rural Affairs-2007*)

Risk class	Genotip *PrP*	Elite farm		Production farms	
Class 1	ARR/ARR	20,79		6,70	
Class 2	ARR/ARQ	22.7	28.65	31,96	33,31
	ARR/AHQ	0.00		0.57	
	ARR/ARH	6.18		0.78	
Class 3	ARQ/ARH	2.25	50.56	2.37	58.68
	ARQ/AHQ	-		0.74	
	AHQ/AHQ	-		0.04	
	ARH/ARH	0.56		0.08	
	AHQ/ARH	-		-	
	ARQ/ARQ	47.75		55.45	
Class 4	ARR/VRQ	0		0.28	
Class 5	AHQ/VRQ	-	0	-	1.03
	ARH/VRQ	0		-	
	ARQ/VRQ	0		1.03	
	VRQ/VRQ	0		-	
	χ^2	25.3874***; L.D.=7; p<0.001		0.9895; G. L.=13; p>0.05	

The individuals with the highest resistance to scrapie, the ones of ARR/ARR type framed in the first risk class (R1), are over three times more common in the elite farm (20.79%) than in the production farms (6.70%).

The sheep included in the second risk class (R2) have a low level of disease development. The animals of this class are by five percents more in the production farms (33.31%) than in the elite farm (28.47%). In production farms all three genotypes of this class are met, but the share is held by individuals with ARR/ARQ genotype (31.96%), the other two genotypes (ARR/AHQ and ARR/ARH) recording very low incidence (less 1%). In the elite farm the ARR/ARQ individuals (22.47%) are also preponderant, but the ARR/ARH individuals have some contribution, too (6.18%). The individuals of ARR/AHQ type missing in elite farm.

In the third risk class (R3) there are included sheep with a medium level of disease development, but they can be promoted to reproduction with the suggestion of correct partner nominations in mating couples depending on the prionic genotype. In this class, most of Botosani Karakul sheep have ARQ/ARQ homozygous genotype, being with about eight percents more common in production farms (55.45%) than in the elite farm (47.75%). In the elite farm two genotypes (ARQ/ARH and ARH/ARH) are still present, and in the production farms appear the genotypes ARQ/AHQ, AHQ/AHQ and ARH/ARH, too; the AHQ/ARH genotype missing. Of these genetic variants only the ARQ/ARH genotype can be taken into consideration but its frequency is very low, and individuals with the others genotypes are sporadically or accidentally encountered in populations, so that overall R3 class the difference of eight percents between the two of farm types remains (58.68%, respectively 50.26%).

In the elite farm the individuals framed in two major risk classes of disease development (R4 and R5) are missing from the prion table. In the production farms individuals within the fourth class (R4), comprising a single genotype (ARR/VRQ), occur with a low frequency (0.28%). Around 1% of individuals present the highest contamination level with prionic agent, the ARQ/VRQ individuals being the only present genotype in this class (R5).

In terms of genetic similarity and differentiation, the genetic distance is very small (0.0254) and the informational correlation coefficient has a very high value (0.9749) between the two populations from the two farm types. Logically, it should have that the genetic distance to be *0* and the informational correlation to be absolute (*1*) between the two populations since they belong to the same breed. But technological specificities of sheep improvement lead to small differentiations of genetic structure at the PrP locus between the two populations (Table 2).

Table 2. Genetic distance (*D*) and informational correlation coefficient ($R_{x,y}$) between the two farm types of Botosani Karakul sheep

Botosani Karakul sheep	D)	$R_{x,y}$
Elite farm Production farm	0.0254	0.9749

From the viewpoint of prionic polymorphism it comes out that in the population of elite farm the allelic frequencies develop a lower informational energy (0.3586) than in the population of private farms (0.4949). As such, the genetic diversity achieves a higher coefficient in the elite farm (0.6414) as compared with the production farms (0.5051). By extension, the heterozygosity is richer in elite farm (0.51) than in the production farms (0.41), too (Figure 2).

The expectation was that the situation to show the opposite of the observed phenomenon because most of the research indicates that the polymorphism restriction (decreasing the allele number) leads to decrease of genetic diversity ([17, 18, 25, 26, 27]. Even more so, as the population of production farms falls in the Hardy-Weinberg law rigors, while the population of elite farm is in very significant genetic disequilibrium at the PrP locus. The only explanation for this given fact in informational-statistical paradigms is a more balanced proportioning among the frequencies of the three alleles in the population of elite farm and a wider gap among the frequencies of the five alleles in the population of production farms. A comparative analysis of the contamination probabilities with prionic agent, it is found that the sheep of elite farm has a more increased resistance to scrapie. Half the population presents a medium risk of disease (R3 class), while the other half includes individuals which have a high (R2 class) or very high (R1 class) resistance to scrapie. The classes with a major risk for scrapie (R4 and R5) are not found in sheep of elite farm. In production farms the individuals with high and very high resistance to scrapie are less frequent with 10% than those of elite farm. Also, the individuals with medium risk of disease are more common by 8%. But in the commercial farms, in addition to the first three risk classes, there are also individuals with high or very high susceptibility to scrapie. Although the incidences of these individuals are quite low (about 1.5%), they present a very high dissemination probability of disease in the next generations, especially because of the VRQ allele presence.

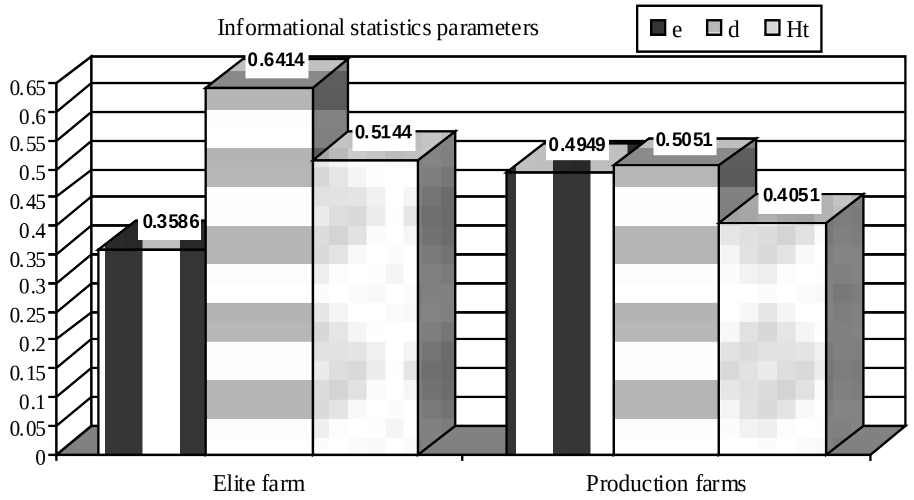


Figure 2. Coefficients of informational energy (*e*) genetic diversity (*d*) and heterozygosity (*Ht*) at the PrP locus in Botosani Karakul sheep depending on the farm type

Overall risk classes, the defense potential of animals on converting the PrP^{c} normal protein in its PrP^{Sc} pathological isomorph is more developed in sheep of elite farm than in individuals of commercial farms. This factual situation could be due to the following reasons:

- the selection criteria are more stringent in the elite farm than in the production farms;
- the artificial selection pressure is more pronounced in elite farm for fixing some production traits in the creation and strengthening process of breeding lines and colour varieties in this sheep breed;
- fixing only certain valuable genotypes in sheep of elite farm can be associated with polymorphism narrowing at the PrP locus through elimination of those genotypes associated with a higher susceptibility to scrapie;
- in the production farms the selection pressure being more relaxed, there are created the expression prerequisites of more prion genotypes, including those associated with low resistance to this disease;
- if in the elite farm the Botosani Karakul sheep are bred in pure breed, in the production farms we deal with an impurification phenomenon of the breed through crossbreeding between Botosani Karakul sheep with Tsurcana sheep, procedures applied by private farmers; the argument seems highly plausible because the Tsurcana breed from the North-eastern of Moldavia is characterized by a relatively significant incidence of VRQ allele (about 7%) [17]. This hypothesis can also be supported by other experimental results obtained in all Tsurcana ecotypes from Transylvania, the traditional breeding basin of this breed. The VRQ allele is present, being rarely met in the Bistrita ecotype (3%) or having a considerable spread in the Hateg and Sibiu ecotypes (9%). Consequently, the VRQ allele enters the genotypic combinations both with ARR allele within R4 class (ARR/VRQ) and especially with ARQ allele within R5 class (ARQ/VRQ). Thus, the genotypes with a high and major risk for scrapie have an important share in the prionic panel of this breed, varying between 1.5% (in the Bistrita ecotype) and 4-6% (in the Hateg and Sibiu ecotypes) within R4 class and between 4.5% (in the Bistrita ecotype) and 12-14% (in the Hateg and Sibiu ecotypes) within R5 class, making from the Tsurcana a vulnerable breed to the prionic agent (28, 29).

The experimental results emphasize the need to apply the most rigorous selection criteria for improving the Botosani Karakul sheep and raising them in a pure breed. They show undoubtedly the genotyping need at the PrP locus of Botosani Karakul sheep and generally of the whole ovine species to prevent this disease in the unaffected flocks or to eradicate the disease where the phenomenon morbid already was installed. In addition, the prion profile features represent very useful tools for achieving the genetic and physiological prophylaxis of sheep populations. Finally, the sheep expertise as regards their resistance to scrapie is very necessary, providing guarantees on animal and man health and creating serious prerequisites for food safety and security in the human community.

4. Conclusions

Using the Real-Time PCR method, the genetic profiles at the determinant locus of scrapie were obtained in the Botosani Karakul sheep bred in two farm types: elite farm and production farms.

Between the two types of breeding farms there are differences of molecular-genetic structure at the PrP locus (number of alleles and genotypes, allele and genotype frequencies) inducing to animals the differentiated predispositions on the susceptibility to prion morbidity in terms of individual framing in risk classes.

The sheep of elite farm are clearly advantaged in the respect of resistance to scrapie as compared to the sheep of production farms; half of sheep from elite farm present a medium risk (R3) of infection with prion agent and the other half of individuals have a high resistance (R2) or very high (R1) to scrapie; the sheep from the major risk classes (R4 and R5) are missing in the elite farm.

The sheep of production farms are more susceptible for contracting the prion disease because the animals with medium risk (R3) are more numerous and those with increased resistance (R1 and R2) are less as compared to sheep from elite farm.

In addition, in the production farms appear also low proportion of individuals belonging to the major risk classes (R4 and R5) representing the vectors for the disease dissemination in the next generations.

The different aptitudinal features relating to the resistance to scrapie would be due to the technological characteristics of sheep improvement in the two farms types: the selection criteria has a strong accuracy in elite farm, while in production farms the selection pressure is more relaxed, also existing the half breeding possibility of Botosani Karakul with Tsurcana.

In terms of informational statistics between the two populations there is a high degree of genetic similarity; contrary to expectations, the genetic diversity is more developed in the population of elite farm than in the population of production farms, the primary role having the dispersional aspect of prion alleles and not their number.

Identification of prion profiles in sheep represent opportunities for achieving the genetic and sanitary veterinary prophylaxis in the farm animals, as well as the traceability of animals and of their productions, thus implementing the concept of food safety and security in human communities.

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