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**Analysis of the *PRNP* gene polymorphisms in healthy
Greek sheep during 2012 - 2016**

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ABSTRACT. Scrapie is a slowly progressive infectious disease of sheep and goats that causes degeneration of the central nervous system. Scrapie is one of several transmissible spongiform encephalopathies (TSEs), like the bovine spongiform encephalopathy (BSE). In sheep, polymorphisms at codons 136, 154 and 171 of the host gene PRPN that encodes the PrP protein, are known to be closely linked to susceptibility or resistance to natural and experimental classical scrapie. In many countries, but not in Greece, breeding programs have been implemented to increase genetic resistance. This study was supported mainly by the private initiatives of farmers willing to improve their flocks by increasing the resistance to scrapie. Thus, the PrP genotypes (of the three mentioned codons) from 5815 blood samples of clinically healthy rams from 160 healthy flocks during the period 2012 – 2016 were determined. Additionally, 1399 blood samples were genotyped only for the 171 codon. Samples were analyzed by Real Time PCR (TaqMan probes) with specific labeled probes. Our results showed an increased percentage of the two genotypes, ARR/ARR and ARR/ARQ linked with resistance to the disease (27.29% and 34.6%, respectively) and relatively reduced percentage of the genotype ARQ/ARQ (24.23%) which is associated with susceptibility to disease and is the most common genotype in the Greek flocks. This joined effort has resulted in the establishment of an important number of farms with an increased population of genetically resistant rams to classical scrapie.

Keywords: classical scrapie resistance/susceptibility, PrP genotypes, breeding programme

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ΠΕΡΙΛΗΨΗ. Η τρομάδης νόσος (scrapie) αποτελεί μια βραδέως εξελισσόμενη λοιμώδης νόσος των προβάτων και των αιγών, η οποία οδηγεί σε εκφυλισμό του κεντρικού νευρικού συστήματος. Η scrapie είναι μια μορφή των μεταδιδόμενων σπογγωδών εγκεφαλοπαθειών (TSEs) όπως και η σπογγώδη εγκεφαλοπάθεια των βοειδών (BSE). Στα πρόβατα είναι γνωστό ότι οι πολυμορφισμοί στα κωδικόνια 136, 154 και 171 του γονιδίου PRPN που κωδικοποιεί τη πρωτεΐνη PrP σχετίζονται άμεσα με την ευαισθησία/ανθεκτικότητα της φυσικής και πειραματικής κλασικής scrapie. Σε πολλές χώρες, αλλά όχι στην Ελλάδα έχουν εφαρμοστεί προγράμματα με στόχο την αύξηση της ανθεκτικότητας στη νόσο. Η παρούσα εργασία στηρίχτηκε κυρίως στην ιδιωτική πρωτοβουλία παραγωγών που ήταν πρόθυμοι να βελτιώσουν την εκτροφή τους αυξάνοντας την ανθεκτικότητα στην τρομάδη νόσο. Έτσι μελετήθηκαν οι γονότυποι του γονιδίου PrP (των τριών προαναφερθέντων κωδικονίων) σε 5815 δείγματα αίματος από υγιή κριάρια προερχόμενα από 160 υγιείς εκτροφές, κατά τη χρονική περίοδο 2012-2016. Επιπρόσθετα σε 1399 δείγματα αίματος αναλύθηκαν μόνο οι πολυμορφισμοί του κωδικονίου 171. Οι γονοτυπικές αναλύσεις πραγματοποιήθηκαν με τη χρήση της Real Time PCR (TaqMan ανιχνευτές) χρησιμοποιώντας ειδικά σημασμένους ανιχνευτές. Τα αποτελέσματά μας παρουσιάζουν αυξημένα ποσοστά των δύο γονοτύπων ARR/ARR και ARR/ARQ, οι οποίοι συνδέονται με ανθεκτικότητα έναντι της νόσου (27.29% και 34.6%, αντίστοιχα) και σχετικά μειωμένο το ποσοστό του γονότυπου ARQ/ARQ (24.23%) που συνδέεται με ευαισθησία έναντι της νόσου και ο οποίος είναι ο πιο κοινός γονότυπος στις Ελληνικές εκτροφές. Αυτή η κοινή προσπάθεια οδήγησε στην καθιέρωση ενός σημαντικού αριθμού εκτροφών που διαθέτουν αυξημένο πληθυσμό κριαριών γενετικά ανθεκτικών στην κλασική scrapie.

ΛΕΞΕΙΣ ΕΥΡΕΤΗΡΙΑΣΗΣ: ευαισθησία/ανθεκτικότητα scrapie, PrP γονότυποι, πρόγραμμα γενετικής βελτίωσης

INTRODUCTION

Scrapie is a slowly progressive infectious disease of sheep and goats that causes degeneration of the central nervous system. The main constituent of the infectious agent is an aberrant isoform (PrP^{Sc}) of the normal cellular (PrP^C) prion protein (PrP), which is a cell-surface glycoprotein [19]. It has been showed that the PrP abnormal prion protein form consists of an approximately 40% of beta sheet folding that transforms it to protease resistant and infectious (Prusiner, 1991). The disease is fatal after a long incubation period and continues to exist within a herd by spread between herdmates or by transmission from ewe to lamb (Goldmann et al., 1990). The transmission as well as the incubation period of the disease depends on the exposure to the infectious agent, the scrapie strain and the genetic background of the host (O'Rourke et al., 1997). Prion protein of sheep is a protein of 256 amino acids. More than 15 polymorphisms of the PRPN gene that encodes PrP of sheep have been reported (DeSilva et al., 2003) but only the polymorphisms at codons 136, 154 and 171 are known to be closely linked to susceptibility to natural and experimental classical scrapie (Bossers

et al., 1996, Hunter et al., 1996, Dawson et al., 1998, Elsen et al., 1999, Thorgeirsdottir et al., 1999, Tranulis et al., 1999). The polymorphisms of the codon 136 (Alanine/Valine/Threonine; A/V/T), codon 154 (Arginine/Histidine; R/H) and codon 171 (Arginine/Glutamine/Histidine/Lysine; R/Q/H/K) have been analyzed in many studies (Goldmann et al., 1994, Hunter et al., 1994, Gloucard et al., 1995). It has been shown that the VRQ haplotype is strictly associated with susceptibility in homozygosis and heterozygosis (Belt et al., 1995, Hunter et al., 1996), while the ARR/ARR genotype is correlated with resistance to scrapie (Goldmann et al., 1994, Hunter et al., 1994, Baylis et al., 2002). This information is used in European countries for the implementation of national breeding programmes to reduce the existed susceptibility to scrapie (Arnold et al., 2002). The haplotype AHQ may be associated with increased resistance and incubation time in some breeds (Hunter et al., 1996, Dawson et al., 1998, Elsen et al., 1999, Thorgeirsdottir et al., 1999, O'Doherty et al., 2002), while it is associated with high susceptibility in purebred and crossbred German Merinoland sheep (Lunken et al., 2004) as well

as in a Romanov flock (Diaz-Avalos et al., 2005). In Greece, Histidine in codon 154 has been found at a significantly high frequency in the Chios crossbred scrapie-affected sheep, suggesting that probably: (a) this allele is associated with increased susceptibility, at least in Chios breed, (b) there is a local scrapie strain strongly correlated with Histidine in codon 154 or (c) there is a combination of the allele susceptibility and the scrapie strain tropism (Ekateriniadou et al., 2007a). The ARQ haplotype's susceptibility varies between sheep breeds, while the ARH and TRQ haplotypes seem to be rather neutral (Dawson et al., 1998, Billinis et al., 2004). Little is known about haplotype ARK and it has not been associated with scrapie resistance or susceptibility (Acutis et al., 2004, Alexander et al., 2005).

In Greece, where the sheep population stands at about 9.5 million, not much is known about PrP alleles' distribution in healthy crossbred sheep. Scrapie was firstly diagnosed in 1986 (Leontides et al., 2000), moreover Billinis et al., (2004) have found some polymorphisms in healthy and scrapie-affected sheep. Ekateriniadou et al., (2007b) have described the alleles and genotype frequencies of healthy sheep from 13 rare breeds as well as from healthy and scrapie-affected sheep in the period 2003-2005 where the ARQ/ARQ genotype was predominated in all three sheep groups tested (Ekateriniadou et al., 2007a). Greece is the EU-country with the second highest incidence of positive scrapie cases after Cyprus (EU- Health and Food Safety 2014). In many countries as mentioned above, but not in Greece, breeding programs have been officially implemented to increase genetic resistance.

The purpose of the current study was to determine the PrP genotypes (codons 136, 154 and 171) from blood samples of clinically healthy rams from 160 healthy flocks during the period 2012-2016. The study was based on the private initiatives of farmers and both freelancers and state veterinarians for the establishment of an important number of farms with an increased population of resistant rams.

MATERIALS AND METHODS

Samples

In total 7214 sheep have been analyzed. The samples originated from 160 Greek healthy flocks of scrapie positive and negative areas. The majority of these flocks (105) originated from Northern Greece, 45 from Central and Western Greece and 10 from Southern Greece. The most farmers sent for genotyping all their males. A lot of these flocks were sending samples each year for genotyping their newborns.

The 5815 samples were fully analyzed by identifying the genotypes of the three amino acids (aa) 171, 136 and 154, while in the 1399 samples only the 171aa was genotyped. These 1399 samples were the newborns from some of these flocks (40) mentioned above that had sent samples more than once and adopted breeding schemes having as a target to increase the genetic resistance to scrapie by keeping only the most resistant to scrapie animals.

Genomic DNA extraction

Genomic DNA was extracted from the blood samples using the PureLink Genomic DNA kit (Life Science) according to the protocol for blood lysate.

Table 1. Taqman probes used for the detection of SNPs at codons 136, 154 and 171 by Real-Time PCR.

Target aa	Sequence (5'-3')	5'-Label	3'-Label
A136	CTCATGGCACTTCCCA	6FAM	BHQ1
V136	CTGCTCATGACACTTCCCAG	HEX	BHQ1
R154	CCGTTACTATCGTGAAAACATGTAC	ROX	BHQ2
H154	CCGTTACTATCATGAAAACATGTACC	Cy5	BHQ2
R171	CCAGTGGATCGGTATAGTAACCA	6FAM	BHQ1
H171	AGACCAGTGGATCATTATAGTAACCA	HEX	BHQ1
Q171	CCAGTGGATCAGTATAGTAACCAGA	ROX	BHQ2
K171	ACCAGTGGATAAGTATAGTAACCAGA	Cy5	BHQ2

PrP genotype analysis

A Real Time PCR method was used to detect the eight polymorphisms: 171Q/R/H/K, 136A/V and 154R/H. The analyses were performed by two tetraplex Real-Time PCR reactions. The first one was for the detection of the four polymorphisms of the 171aa and the second one for the polymorphisms of the 136aa and 154aa. In both tetraplex reactions a DNA fragment of 180bp was amplified using the primers: ScF-5'-GCC TTG GTG GCT ACA TG-3' and ScR-5'- CTG TGA TGT TGA CAC AGT CAT-3'. The sequences of the labelled probes used for the detection are shown at Table 1. Amplification reaction mixtures were prepared at a final volume of 15 µl containing 1X KAPA PROBE FAST qPCR kit Master Mix Universal, 400nM of each primer and probes and 40-50 ng of sample's DNA. qPCRs were performed in a Chromo4™ Real-Time Detector. The cycling conditions for all the reactions consisted of the initial denaturation at 95oC for 3min, and 45 cycles of denaturation at 95oC for 3sec and annealing/extension at 62oC for 30sec. The method has been validated by applying it in samples that the PrP gene has been sequenced and also in control samples sent by the European Reference Lab (AHVLA). In case of double heterozygosis, the genotypes order e.g. ARR/AHQ and not AHR/ARQ, has been determined following the *PRPN* genotypes' order that has been recorded through the relevant literature.

Statistical analysis

The results were analysed statistically using the Chi square test to compare the frequencies of the Types 1, 2 and 3 of the EU Scrapie Plan during 2012-2016.

RESULTS

PrP allelic variants

The allelic variants in codons 136 (A/V) and 154 (R/H) were genotyped in 5815 healthy rams. In codon 136, Alanine was detected with a frequency of 99.61% and Valine with a frequency of 0.39%, having the A/A homozygotes predominated (99.3%) over the V/V homozygotes (0.03%), appeared in only 2 samples. As far as codon 154 is concerned, Arginine was the predominant amino acid (96.1%) with H/R heterozygotes found with a frequency of 7.2%.

Table 2. Frequency of the PrP alleles in codons 136, 154 (n=5815) and 171 (*n= 7214) detected in healthy crossbred sheep

PrP Codon	Allelic variant (aa)	Frequency(%)
136	A	99.61
	V	0.39
154	R	96.1
	H	3.9
171*	Q	50
	R	47.26
	H	2
	K	0.74

The tetramorphism Q/R/H/K in codon 171 was genotyped in 7214 healthy males and Glutamine was the predominant amino acid detected at the 50% of the samples. Arginine was detected in a high frequency (47.26%) not only in heterozygotes but also in homozygosis (26.84%), while Histidine and Lysine were detected in the very low frequencies of 2 and 0.74%, respectively. The frequency distribution of the alleles is shown in Table 2.

PrP haplotype variants

In the 5815 analysed samples, 9 known haplotypes have been appeared. The most frequent haplotype was ARR with a frequency of 47.78%. The ARQ haplotype has been found in a very high frequency (44.93%), while the AHQ and ARH haplotypes were detected with a frequency of 3.86% and 2.24%, respectively.

Table 3. Frequency distribution of the PrP haplotype variants detected in n=5815 healthy crossbred sheep.

Prp haplotypes	Frequency (%)
ARR	47.78
ARQ	44.93
AHQ	3.86
ARH	2.24
ARK	0.75
AHR	0.02
AHH	0.03
VRQ	0.36
VRH	0.03

The 5 rest haplotypes (ARK, AHR, AHH, VRQ and VRH) were the less frequent detected in very low frequencies varying from 0.75 to 0.02%. The frequency distribution of the PrP haplotypes is shown in Table 3.

PrP genotypes

The 9 different haplotypes resulted in 23 genotypes of the 5815 analysed samples. These genotypes were categorized into 5 Types according to the level of resistance/susceptibility to scrapie as defined by the EU Scrapie Plan. Type 1 is comprised only by the ARR/ARR genotype and is the most resistant to scrapie. Type 2 is comprised by the genotypes ARR/ARQ, ARR/AHQ, ARR/ARH, ARR/ARK and AHR/AHQ

Table 4. Classification and frequency the 23 PrP genotypes detected in 5815 health crossbred sheep based on the EU Scrapie plan.

Type	Degree of resistance	Genotype	Frequency (%)
1	The most resistant	ARR/ARR	27.29
2	High level of resistance to scrapie	ARR/ARQ	34.6
2		ARR/AHQ	3.37
2		ARR/ARH	2.05
2		ARR/ARK	0.69
2		AHR/AHQ	0.03
3	Susceptible / Little resistance to scrapie	ARQ/ARQ	24.23
3		ARQ/AHQ	3.59
3		ARQ/ARH	2.15
3		ARQ/ARK	0.67
3		AHQ/AHQ	0.29
3		ARH/AHQ	0.05
3		ARK/ARK	0.02
3		ARH/ARK	0.05
3		ARH/ARH	0.09
3	AHQ/ARH	0.07	
3	AHQ/ARK	0.03	
4	Susceptible to scrapie not to be used for breeding	ARR/VRQ	0.29
4		AHQ/VRQ	0.03
5	Highly susceptible to scrapie, not to be used for breeding	ARQ/VRH	0.05
5		ARQ/VRQ	0.31
5		ARK/VRQ	0.02
5		VRQ/VRQ	0.03

presenting high level of resistance to scrapie, while Type 3 is represented by the ARQ/ARQ, ARQ/AHQ, ARQ/ARH, ARQ/ARK, AHQ/AHQ, ARH/AHQ, ARK/ARK, ARH/ARK, ARH/ARH, AHQ/ARH and AHQ/ARK genotypes presenting no resistance to scrapie. Finally, Type 4 is represented by the genotypes ARR/VRQ and AHQ/VRQ and Type 5 by the ARQ/VRQ, ARQ/VRH, ARK/VRQ and VRQ/VRQ genotypes which are the most susceptible to scrapie and completely unsuitable for breeding schemes.

The most frequent genotype was ARR/ARQ (34.6%) which is related with a high level of resistance to scrapie. The ARR/ARR genotype that is considered as the most resistant to scrapie was present in a frequency of 27.29%. The ARR/- genotypes associated with resistance to the disease had a total frequency of 68.29% while the susceptible to scrapie ARR/VRQ, ARQ/VRQ and VRQ/VRQ genotypes were found in a frequency of 0.29%, 0.31% and 0.03%, respectively. ARQ/AHQ/- and ARH/- genotypes as well as VRQ/- genotypes (the three mentioned above genotypes plus the ARK/VRQ and AHQ/VRQ genotypes) that are associated with high susceptibility to scrapie, were found in a frequency of 31.05%, 7.41%, 4.41% and 0.68%, respectively. Table 4 lists the genotypes and their frequencies.

The analysis performed showed a significant increase ($p < 0.01$) in Type 1 (from 14.53% to 22.67%) and Type 2 (from 26.5% to 41.28%) genotypes along with a significant decrease ($p < 0.01$) in Type 3 (from 58% to 35.11%) genotypes during the years 2012-2016. The Type 4 and Type 5 genotypes' frequencies were too low constantly with no specific trend (Figure 1).

In the genotype analysis of the 171 codon in the 1399 samples, a significant increase ($p < 0.01$) of Arginine frequency was also observed both in homozygosis and heterozygosis from 14.88 to 33.77% and from 28.84 to 47.49%, respectively, while a significant decrease ($p < 0.01$) was observed in the frequency of Glutamine both in homozygosis and heterozygosis from 53.95 to 17.28% and from 2.33 to 1.46%.

DISCUSSION

Scrapie of small ruminants remains a problem in Greece since no national breeding programme has been implemented. The aim of the present study was

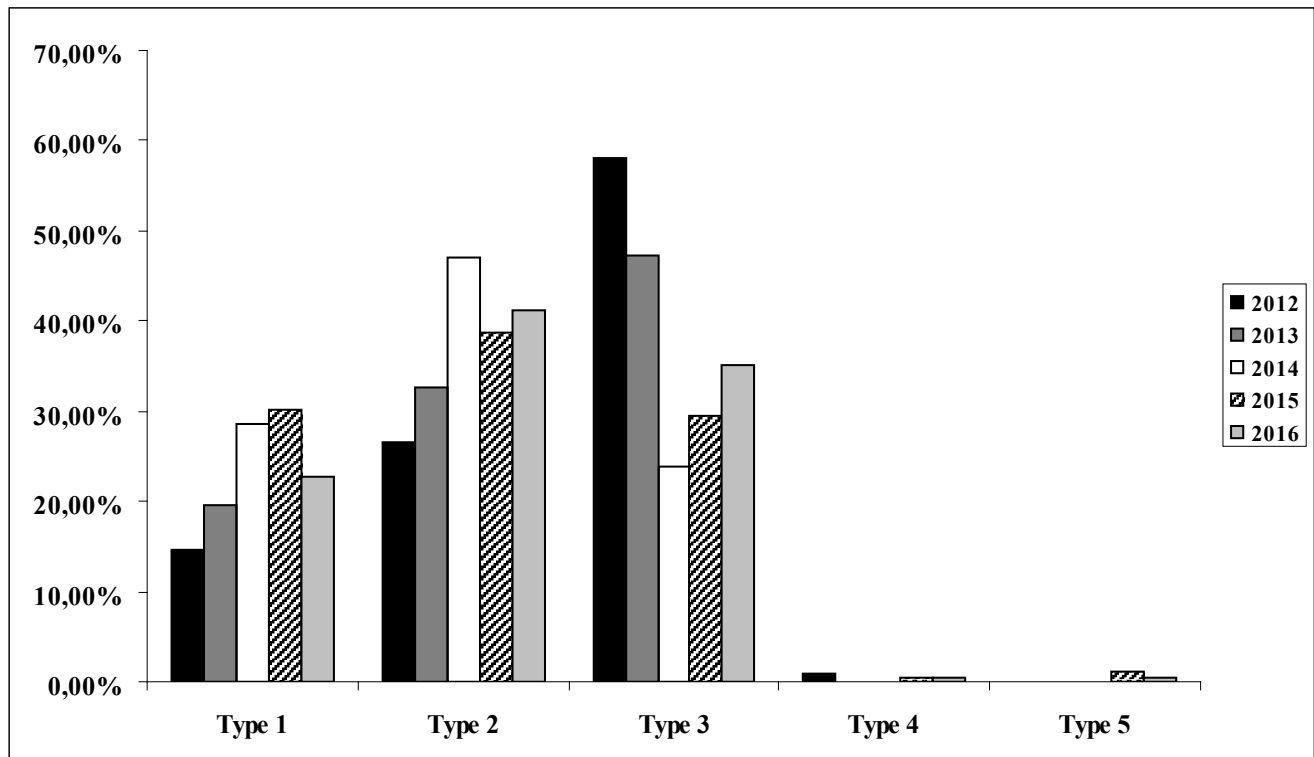


Figure 1. Distribution of scrapie resistant/susceptible Types based on the EU Scrapie Plan classification, detected during the time period 2012-2016

to determine the genotypes of the three codons of the PrP protein linked to resistance/susceptibility to scrapie in healthy Greek sheep flocks.

The results showed the frequencies of the alleles, the haplotypes, as well as those of the genotypes, for the PrP codons 136 and 154 in 5815 samples and for the codon 171 in 7521 samples. The analyses showed not only the existence of the most frequent haplotypes ARR, ARQ, AHQ, ARH, VRQ (Elsen et al., 1999, Thorgeirsdottir et al., 1999, O'Doherty et al., 2002, Billinis et al., 2004) but also the ARK haplotype, a rare haplotype that has been detected in Italian Bielese breed with a frequency of 2.5%; (Acutis et al., 2004), Mongolian Khalkh and Sartuul with a frequency 0.6%; (Gombojav et al., 2003), in one Spanish animal (Acin et al., 2004), in Oklahoma at 0.35%, in Kivircik sheep breed in Turkey at 0.35% (Oner et al., 2011) as well as in Greek purebred and cross-bred animals at 1.6% (Billinis et al., 2004). Two other rare haplotypes, AHH and VRH were detected both in the very low frequency of 0.03%. In the 9 haplotypes found in the samples, ARR was the predominant one in all flocks (47.78%) being followed by haplotype

ARQ (44.93%). The AHQ haplotype was found in low frequency (3.86%), lower than the frequency that has been detected in a previous study (Ekateriniadou et al., 2007b) in rare Greek breeds (6.31%). The rare ARK haplotype was also detected in a low frequency of 0.75%. The VRQ haplotype associated with susceptibility to scrapie was also detected at a very low frequency (0.36%), whereas the neutral haplotype ARH was found in a frequency of 2.24%.

The analysis of the 5815 samples revealed 23 different genotypes. The ARR/ARQ genotype (Type 2) was the predominant one with a frequency of 34.6%. The ARR/ARQ genotype is also appears as the most frequent genotype in other countries/breeds that have begun an effort to increase the resistance to scrapie, such as the Sicilian sheep with a frequency of 39.9% (Reale et al., 2015) and the Polish Pomeranian Landrace sheep with a frequency of 35.9% (Proskura et al., 2013). The second most frequent genotype was the resistant genotype to scrapie (Type 1) ARR/ARR (27.29%) while the most frequent Greek genotype ARQ/ARQ (Type 3), was the third most frequent genotype (24.23%).

The analyses during the period 2012-2016 showed a significant increase ($p < 0.01$) of the scrapie resistant genotypes (Types 1 and 2) with a parallel decrease in the scrapie susceptible (Type 3) genotypes (Figure 1). The increasing trend of Type 1 and Type 2 genotypes disrupted presenting a small decrease in 2016 and in 2015, respectively, accompanied with a slight increase of Type 3 genotypes during 2015-2016 (Figure 1). These results could be attributed to the analyses of the newborns samples from 40 flocks that had sent samples more than once and adopted breeding schemes, focusing in the analysis of the 171aa. The analyses of these samples as mentioned above, presented a significant increase ($p < 0.01$) in the frequencies of Arginine and a significant decrease ($p < 0.01$) in the frequencies of Glutamine in both homozygosis and heterozygosis. A decrease of the allelic variants RR and R/- was observed in 2014 due to the small number of samples analyzed only for the 171aa since the newborns genotyped in 2012 and 2013 needed at least a year for introducing them to the reproducing procedure.

Finally, the most susceptible to scrapie Types 4 and 5 genotypes were detected during this time period at very low frequencies.

The farmers along with the freelancers and state veterinarians successfully followed the suggestions for selection breeding against scrapie based on the *PRPN* analyses performed during 2012-2016. The conversion time of a flock to scrapie resistant is depending on the number of the scrapie resistant rams and ewes and on the level of scrapie resistance that the breeding scheme was begun. Arnold's model (Arnold et al., 2002) suggests that it will take at least 20 years to ensure that all slaughter lambs carry at least one ARR allele.

CONCLUSION

The initiative of the farmers and private and state veterinarians to genotype their future breeders began a significant effort for the transformation of their flocks from scrapie susceptible to scrapie resistant.

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