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Genotyping of some scrapie-positive sheep cases and scrapie affected flocks in Bulgaria in 2008–2010

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Abstract
Scrapie is a genetically associated neurodegenerative disease caused by the accumulation of an abnormal protein. The aim of this study was to perform genotyping of some scrapie-positive cases in sheep and the flocks they come from and to compare the effect of specific polymorphisms. It is important to examine positive cases in order to determine the possible polymorphism in the *PRNP* gene and its possible phenotypical appearance. Seventy-nine blood samples and 7 brain samples from scrapie affected flocks were tested. Five flocks of sheep, of different size and breed, were genotyped and the results showed that 3 of the animals in flock 1 had additional polymorphism in codon 138, nucleotide 413. In two of the animals from flock 5 there was homozygous and heterozygous polymorphism in codon 138, position 414. The VRQ allele, which is associated with high susceptibility to scrapie, was found in all the flocks, except flock number 2. Our data showed that the atypical scrapie cases are not associated with polymorphism in codon 141. Additional polymorphism was also observed at codons 137, 138, 151 and 152. Strategies for eradication of scrapie have been employed.

1. Introduction

Transmissible spongiform encephalopathies (TSE) are a group of neurodegenerative diseases caused by the accumulation of an abnormal protein (PrP^{sc}), which is an isoform of a normal cellular protein (PrP^{c}) [14, 29]. Similar etiology is seen in humans in Creutzfeldt–Jakob Disease, Gerstmann–Sträussler–Scheinker syndrome, Kuru and variant Creutzfeldt–Jakob Disease, as well as Bovine Spongiform Encephalopathy, Chronic wasting disease and TSE in carnivores. In sheep and goats, the disease has been known as scrapie since the 18^{th} century [24, 27]. The disease became spread worldwide in the 20th century, especially after World War II, through export of British sheep in incubation period [8]. The disease is not genetic, but there is genetic predisposition associated with polymorphism in codons 136, 154 and 171 of the *PRNP* gene for the

classical scrapie in sheep, and 141 codon for the atypical form of the disease [11, 13, 15, 17, 25]. Valine in codon 136, arginine in codon 154 and glutamine and histidine in codon 171 determine susceptibility to the disease [2, 13]. The VRQ combination of these codons is associated with high susceptibility to scrapie, while the ARR allele is associated with resistance to the disease [15].

Some authors have reported polymorphism in other codons of the *PRNP* gene as well, like codon 83, 101, 112, 116, 127, 137, 138, 151, 172, 175, 176, 180, 189, 195, 196, 211, 231, 237 and 241, but their significance for scrapie disease is neutral or undefined [35, 36, 39].

Having in mind that the causative agent of scrapie, the Bovine Spongiform Encephalopathy and the zoonotic variant Creutzfeldt–Jakob disease in humans may interact in unpredictable ways poses the need to continue research efforts to restrict their spread.

During 2008-2010 years, approximately 1 400 000 million sheep were bred in Bulgaria, per year. Private farms usually have from 5 to 20 sheep. In some rural economies the number can reach 300 to 500. Only a few flocks have 1000 animals or more and in private farms and smaller rural economies sheep and goats are often bred together.

The aim of the present study was to perform genotyping of some scrapie-positive cases in sheep and the flocks they come from and to compare the effect of specific polymorphisms.

2. Materials and Methods

Diagnostic method - ELISA

Two different kits were used, more precisely, ELISA TeSeE Purification and Detection Kits (Bio-Rad, France) for all samples but for the year 2010, when Enfer TSE Version 3 (Enfer Scientific, Ireland) was used. The manufacturers' instructions were followed.

Confirmatory method – Western Blot (WB)

TeSeE™ Western Blot Kit (BioRad, France) was used for confirmation of ELISA positive samples, according to the manufacturers' instructions.

Samples and DNA extraction

Seventy-nine blood samples and 7 brain samples were tested from the flocks affected with scrapie. One of the scrapie cases from 2009 was not included in this examination. Samples were received in the laboratory, according to Directive 999/2001 EC, Annex X, Chapter C, point 3.2(b) [31]. Based on the available information, the present research included 6 cases in the period 2008–August 2010 and a case from 2012 due to its relation with the other cases from 2010.

For isolation of DNA a commercial kit Illustra blood genomic Prep Mini Spin Kit (GE Healthcare, UK) was used following the manufacturer's instructions. DNA was obtained from brain samples through DNeasy Blood & Tissue kit (Qiagen,USA). PCR, genotyping and determination of genotype frequency

DNA was amplified by PCR using the following pairs of primers (kindly provided by AHVLA,Weybridge, UK): Forward 5' – CATTTGATGCTGACACCCTCTTTA – 3' and Reverse 5' – ATGAGACACCACCACTACAGGGCT – 3'. Reactions were conducted in a volume of 25 μL, using Illustra puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK) by Thermocycler QB - 96 (LKB). The PCR procedure described by AHVLA was used.

The obtained amount and quality of DNA was controlled spectrophotometrically with Jenway apparatus (Genova, UK) and by gel electrophoresis – 2% Agarose (GE Healthcare, UK), 10 mg/ml ethidium bromide (Sigma, USA), 100 bp DNA ladder (GE Healthcare, UK), 120 V, 45mA for 45 min have been used.

The genotyping procedure was performed as described before [32]. Data analysis was carried out with MEGA4 computer software [34]. Genotype (f_{ij}) and allele (p_i) frequencies were calculated as follows [12, 19, 38]:

$f_{ij} = n_{ij} / N$ *and* $p_i = (2f_{ii} + \Sigma f_{ij}) / 2$, where n_{ij} is the number of animals with the ij genotype, f_{ij} and f_{ii} is the heterozygous and homozygous genotype frequency, respectively, p_i is the allele frequency and N is the total number of animals.

3. Results

In the National Reference Laboratory "TSE" Bulgaria, 23 344 brain samples from sheep and goats were examined in the period between 2008 and August 2010 and, as a result, 7 cases of scrapie were diagnosed in sheep by rapid tests and were confirmed by Western Blot (Fig.1). There was no case with clinical signs. Farms with confirmed cases of scrapie were with flocks of different sizes and breeds, and the results of the genotype of positively confirmed cases are presented in table 1.

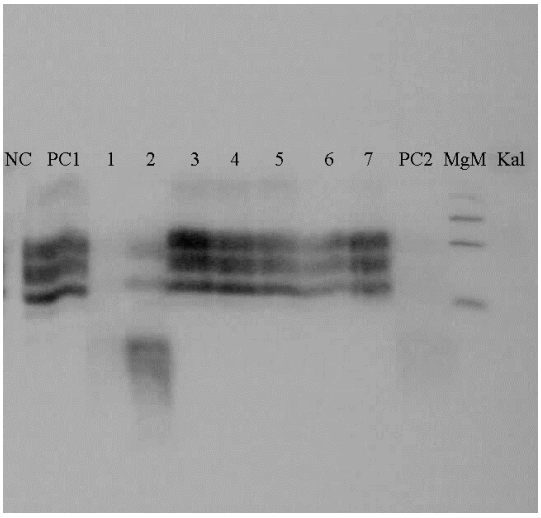

Figure 1. *Confirmation of scrapie-positive cases in sheep included in this study in 2008–2012. NC – negative control; PC1 – positive control of classical scrapie; PC2 – positive control of atypical scrapie; MgM – MagicMark™ XP Western Protein Standard (Invitrogen, USA); Kal – Kaleidoscope (Bio-Rad, France); 1 – 3462, atypical; 2 – 9545 atypical; 3 – 12098, classical; 4 – 5220, classical; 5 – 155, classical; 6 – 808, classical; 7 – 1472, classical;*

Flock number one consists of a local breed and the second flock, of Middle Rodopean breed (sheep from the local breed do not possess the specific features of a particular breed, but are rather a result of cross-breeding). The breeds from the other flocks are shown in table 2, where data from the genotypic breed alignment are presented. What is typical for flock number 5 is that for a period of 45 days 2 cases of ovine scrapie were diagnosed.

The examinations of the allele allocation within the affected flocks are presented in table 3.

In 3 of the animals in flock 1 we observed additional polymorphism in codon 138, nucleotide 413, where Guanine was replaced by Adenine (G→A). This was found to result in a homozygous change of the amino acid Serine to Asparagine in two of the animals and a heterozygous change in one animal. We found homozygous and heterozygous polymorphism in codon 138, position 414, in two of the animals from flock 5, where Cytosine was replaced with Thymine (C→T), i.e. a synonymous change. In this flock, one of the animals was shown to have heterozygous polymorphism in codon 152, as a synonymous change, in position 456, where C→T (Table 4).

Table 1. *Scrapie affected flocks with genotype of scrapie-positive cases and number of investigated sheep*

Flocks	Number of cases/lab. code	Type / age(months)	Genotype of scrapie-positive cases	Breed	Year	Number of investigated sheep[b] /animals in flock
1	1/3462	atypical / 30	ALRR/ALHQ	local	2008	5 / 5
2	1/9545	atypical / unknown	ALRR/ALRR	local + Tzigay	2008	14 / 350
3	1/12098	classical / 18	No investig.	Synthetic population Bulg.sheep	2009	29 / 29
4[a]	1/5220	classical / 52	ALRQ/ALRQ	local	2009	0 /1
5	3 / 155	classical / 35	ALRQ/ALRQ	local	2010	31 / 324
	/ 808	classical / 36	ALRQ/ALRQ	local		
	/ 1472	classical / 84	No investig.	local	2012	0 / 254

[a] Private person's sheep; [b] The positive samples are not included

Table 2. *Breeds of scrapie affected flocks and frequency distribution of different genotypes of the PRNP gene in codon 136, 154 and 171*

flock	breed	Genotype Frequency % And Number of Animals ()				
		ARR/ARR	ARR/AHQ	ARR/ARH	ARR/ARQ	AHQ/AHQ
		R.g. 1	R. g. 2	R. g. 2	R. g. 2	R. g. 3
1	Local	-	-	-	-	-
2	Middle Rhodopean	14.29 (2)	-	-	50.00 (7)	-
3	Synthetic population Bulgarian sheep	12.00 (3)	4.00 (1)	4.00 (1)	32.00 (8)	8.00 (2)
	Mouton Sharole	-	-	-	100 (3)	-
	Ile-de-France	100 (1)	-	-	-	-
	Total distribution	13.79 (4)	3.45 (1)	3.45 (1)	37.93 (11)	6.9 (2)
5	Local	-	-	-	18.18 (4)	-
	Blackhead Pleven	14.29 (1)	-	-	42.86 (3)	-
	Local Stara Zagora sheep	-	-	-	50.00 (1)	-
	Total distribution	3.23 (1)	-	-	25.81 (8)	-

Table 2. *Continued*

flock	breed	Genotype Frequency % And Number of Animals ()				
		AHQ/ARQ	ARQ/ARQ	ARR/VRQ	AHQ/VRQ	ARQ/VRQ
		R. g. 3	R. g. 3	R. g. 4	R. g. 5	R. g. 5
1	Local	-	80.00 (4)	-	-	20.00 (1)
2	Middle Rhodopean	7.14 (1)	28.57 (4)	-	-	-
3	Synthetic population Bulgarian sheep	16.00 (4)	8.00 (2)	4.00 (1)	8.00 (2)	4.00 (1)
	Mouton Sharole	-	-	-	-	-
	Ile-de-France	-	-	-	-	-
	Total distribution	13.79 (4)	6.9 (2)	3.45 (1)	6.9 (2)	3.45 (1)
5	Local	9.09 (2)	63.64 (14)	-	-	9.09 (2)
	Blackhead Pleven	-	28.57 (2)	-	-	14.29 (1)
	Local Stara Zagora sheep	-	50.00 (1)	-	-	-
	Total distribution	6.45 (2)	54.84 (17)	-	-	9.68 (3)

[a] All investigated animals are LL homozygous in codon 141; R. g. - risk group
[b] Mouton Sharole and Ile-de-France consist of rams in flock number 3

Table 3. *Genotype frequency of different allels variants of PRNP gene for 136, 154 and 171 codons*

flock	Allele %				
	ARR	**ARQ**	**AHQ**	**ARH**	**VRQ**
1	-	90	-	-	10
2	39.29	57.14	3.57	-	-
3	37.86	34.485	18.975	1.725	6.9
5	16.135	74.19	3.225	-	6.45

Table 4. *Additional polymorphisms in the PRNP gene of scrapie affected flocks in Bulgaria*

Flock / Number of investigated animals	genotype
1 / 6	$AN^{138}RQ/ AS^{138}RQ$ $AN^{138}RQ/ AN^{138}RQ$ $AN^{138}RQ/ AN^{138}RQ$ $AS^{138}RQ/ AS^{138}RQ$
5 / 33	$AS^{138}RQ/ AS^{138}RQ$ $AY^{152}HQ/ AY^{152}RQ$

*The positive scrapie cases are included

4. Discussion

Management and control of TSE in Europe is laid down in Directive 999/2001 EC and the rules for implementing breeding programs by member states to combat scrapie disease are specified in Decision 2003/100/EC of the European Commission. The importance of breeding programs to combat the disease is essential because there is no implemented treatment in practice and the pathogenesis of the disease and the role of genetic predisposition for its development are not yet fully understood. In this context, the main objective of breeding programs is to increase the frequency of occurrence of the ARR allele and to reduce VRQ allele to its complete disappearance. It is important to note that the selection of scrapie-resistant animals has no direct adverse effect on the most commonly measured characteristics of production [33], as the genes responsible for scrapie susceptibility are located in chromosome 13 [3].

As shown in table 1, three of the classical scrapie cases were found to be homozygous for the allele ARQ, which is associated with susceptibility to scrapie [6]. A homozygous ARR allele and a heterozygous ARR allele in combination with the AHQ allele were observed in atypical scrapie cases. In all the scrapie cases we examined, the animals were LL homozygous in codon 141. These data are in accordance with the results of Moum et al. [25] and Lühken et al. [23], who also report the resistance allele ARR in the classical type of scrapie and the AHQ allele associated with lower susceptibility in sheep affected by atypical scrapie.

The high-susceptibility VRQ allele was found in all the flocks studied by us, except flock number 2. The AHQ allele, which is associated with high resistance and a longer incubation period [26, 35], and the ARH allele, which is defined as neutral [7], were also observed.

These data show that susceptibility of a certain genotype most probably depends on breed belonging.

VRQ/VRQ is a rare genotype; it is mostly found in scrapie affected sheep [16] and is associated with clinical signs of scrapie disease [12]. Unlike other authors [35], we did not find the VRQ/VRQ genotype in scrapie affected flocks. This is most probably due to the small number of tested animals and the low frequency of occurrence of this genotype among Bulgarian sheep [32]. This is probably the reason for not having scrapie cases with clinical signs in Bulgaria. The diversity in genotypes in flock 3 is determined by the breed named Synthetic population Bulgarian sheep (SPBS), which is the basis of the so-called local sheep. This confirms our previous observation [32] showing the range of genotype versions within this breed. This polymorphism is most probably the reason for scrapie cases in local sheep as well as in SPBS, as shown in table 1.

Thorgeirsdottir et al. [35] reported additional polymorphism in the tested scrapie affected flocks, concerning codons 137, 138 and 151. We did not find polymorphism in codons 137 and 151 in our samples, but we identified polymorphism in codons 138 and 152.

The role of nonsynonymous mutations in mammals is linked to thermodynamic stability of mRNA and its secondary structure and has a phenotypic effect and significance for the possibility to prolong cellular half-lives [4, 5, 10, 18, 20]. The importance of nonsynonymous mutations is undeniable, which is why it cannot be excluded that additional polymorphism (synonymous and nonsynonymous), apart from that established in codons 136, 141, 154 and 171, might also possibly play a role in the pathogenesis and the level of scrapie development.

In case of classical scrapie in a flock, Annex VII, point 2.3 (b) (i) or (ii) or point 5 (a) of Directive 999/2001 EC should be applied, generally associated with the destruction of animals; only point 2.3 (b) (ii) gives the opportunity to preserve animals genotyped with the ARR allele without a VRQ allele.

In herds 1 and 2 atypical scrapie was proven, which does not impose restrictions for selection under Directive 999/2001 EC. Because of the need to increase animals carriers of the ARR allele for selection of scrapie-resistant flocks according to EU Directive 999/2001, it would be appropriate for the sheep carriers of genotype ARQ/VRQ to be excluded from breeding schemes, and all other sheep carriers of ARQ/ARQ genotype and their descendants to be bred with material from rams which are ALRR homozygous.

Within the second-generation offspring, an individual ARR/ARQ carrier was excluded from breeding with the aim to select only ARR homozygotes. In flock 2, the 9 sheep from the first and second risk group were used for breeding, and the remaining five animals from the third risk group were

excluded from breeding. Carriers of ARR/ARQ genotype were removed from the first generation here.

In flock 3, 4 and 5 we confirmed classical scrapie cases, in which case Annex VII, point 2.3 (b) (ii) of Directive 999/2001 EC is applicable. Based on the genotyping results in flock 3 it was appropriate to separate the animals from the third, fourth and the fifth risk group and to exclude them from breeding, as well as those from the second group at risk (carriers of ARR/AHQ and ARR/ARH genotype) in order to avoid derivation of a third-generation risk group.

It is interesting that for the period of one month and a half scrapie cases were found in sheep and goats in flock number 5. The animals are kept in one yard, where the newborns stay until they grow up. Having this in mind, as well as the fact that the causative agent of scrapie can be transmitted through the colostrum, milk and placenta [1, 21, 22, 28, 30, 37] and that there is high frequency of the ARQ allele in this flock, we can assume that all these factors have led to the spreading of scrapie disease among sheep.

Flock 5 had 324 animals in 2010 and 254 animals in 2012. In 2009, 31 samples were sent for genotyping, which was 9.57% of the total number of animals in the herd. According to Annex III, point 5, 121 animals over 18 months old should be slaughtered and examined in a farm with 350 animals. Despite all the implemented measures in scrapie infected flocks under Directive 999/2001 EC, there was a new case of the disease in 2012. This was most likely due to the fact that the sheep was 84 months old, and the selection program was probably not applied (this was not required at the time). Another factor that may have additionally contributed is also the presence of an animal in incubation period, which is about 2–5 years [9] that remained outside the group of 121 animals intended for killing and examination.

The atypical scrapie cases are not associated with polymorphism in codon 141. Additional polymorphism was established in codons 137, 138, 151 and 152 but the data we obtained could not give us more information concerning the susceptibility to scrapie disease.

This indicates that even with the application of all measures under Directive 999/2001 EC, it is possible to omit an infected animal. Thus, it is necessary to first genotype the animals, and then, based on the results, to determine which of them should be killed and examined. Then, all the other animals could be used to conduct the breeding program.

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