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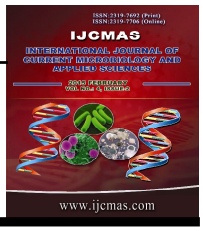

Original Research Article

PrP gene polymorphism and its variations among Bulgarian indigenous sheep breeds

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A B S T R A C T

The aim of the present study was to determine the level of Scrapie resistance among four indigenous Bulgarian sheep breads, based on the three polymorphic PRNP gene codons – 136, 154 and 171. The obtained data showed five PRNP alleles - ARR, ARQ, VRQ, AHQ and ARH, with different prevalence among all breeds. The five alleles resulted into six genotypes ARR/ARR, ARR/ARQ, ARR/ARH, ARQ/ARQ, AHQ/ARQ and ARR/VRQ, each of which belongs to a different Scrapie risk group. The highest frequency of the resistant genotype ARR/ARR was observed among the Stara Zagora breed (20%), while the other breeds showed even lower levels of this important genotype. In addition to the low prevalence of the resistant genotype, we observed relatively high number of susceptible animals carrying some of the riskiest alleles. Twenty two percent of the Northwest Bulgarian merino sheep were carriers of the ARR/VRQ genotype, followed by the Stara Zagora sheepbreed (20%). The target genotype for atypical Scrapie ARQ/AHQ was presentbetweenall breads with highest frequency within the Karakachan breed - 51%. The presence of alleles VRQ and AHQ undoubtedly indicates the lack of appropriateScrapie eradication program inBulgariaand the risk for our sheep industry.

Introduction

Scrapie is neurodegenerative disease affecting small ruminants, known for its long incubation period and specific infectious agent based on a protein molecule – Prion (PrP). The disease was firstly described more than 260 years ago in Britain (Plummer, 1946) and belongs to a group of similar diseases known as Transmissible spongiform encephalopathies (TSE) affecting the central nervous system in both animals and humans (Prusiner, 1998). Interestingly the PrP molecule is found in both healthy (PrP^{C}) and sick animals (PrP^{Sc}). Although the amino acid sequence of both molecules is identical, the secondary protein structure is different which makes the PrP^{Sc}

aggressive and somehow able to transforms the normal cellular prion protein (PrP^C) of the cells. The most common transmission of the pathogen is via peripheral contamination either through oral route or skin lesions (Keulen et al., 2008). Clinical signs include nervousness, fears from light, aggressiveness etc. Typical Scrapie signs are higher skin sensibility, in result of which the animal scratches different parts of its body, salivation, impaired vision etc. (Konold et al., 2010).

The gene encoding the cellular prion protein (PrP^C) is located in sheep chromosome 13 and is called PRNP. The PrP molecule is formed by 256 amino acids, encoded by 768 nucleotides from the PRNP gene. So far polymorphism has been found in 29 codons, three of which are considered as strongly related to the classical disease – 136, 154 and 171. Codon 136 could be either GCC or GTC – encoding the amino acids Alanine (A) or Valine (V), respectively. Hunter and Goldmann(1994) state that the codon for Valine at this position leads to susceptibility, while the Alanine codon leads to resistance. Codon 154 also has two polymorphic states CGT or CAT – encoding the amino acids Arginine (R) or Histidine (H), where Arginine is considered resistant and Histidine is considered susceptible (Laplanche et al., 1993). The third polymorphic codon (171) could be CAG, CAT or CGG, encoding the amino acids Glutamine (Q), Histidine (H) or Arginine (R), respectively. Hunter et al. (1997) state that Glutamine and Histidine codons lead to susceptibility, while the Arginine codon is considered as highly resistant to the disease. All combinations among these codons are still not discovered, but the most common ones are VRQ, AHQ, ARH, ARR and ARQ. Ulvund(2006) organizes all codon combinations into 5 Scrapie risk groups. Most authors (Elsen et al., 1999; Baylis et al., 2002) agree on the thesis that the ARR/ARR genotype is most resistant to the disease, which redound this genotype to be classified into group one (highly resistant) of the National Scrapie plan of Great Britain. On the other hand genotypes AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ belong to group 5 (high susceptibility) (DEFRA, 2001).

Modern genetics gives us the possibility for rapid genotyping and subsequent selection of animals after the ARR/ARR genotype. Researchers from all around the world have investigated the PRNP variations among different sheep breeds and flocks. Tongue et al. (2004) discovered that 82% of British sheep carry the most susceptible allele VRQ, while the level of the ARR allele was under 10%. This data explains the dramatic spreading of the disease in the United Kingdom.

Similar study was performed in Germany (Drögemüller et al., 2004). Based on the observed allele frequencies, authors assume that it will take six to nine generations of selection (depending on the breed) to achieve 99% of ARR homozygous animals.

The easiest way for ARR allele increase is via selection of homozygous rams. Authors from the Netherlands (Windig et al., 2004) investigated local breeds and established that this strategy is inapplicable for small populations, due to the high risk of inbreeding. For small breeds authors suggest initial use of heterozygous for the ARR allele rams and ewes carriers of any allele except for the VRQ. Ones the ARR allele has reached certain rates, the process could be continued with rams homozygous for the ARR allele.

The importance of this polymorphic gene, motivates authors from all around the globe

to investigate the levels of each allele among different breeds. Our country makes its first steps in this field with the investigation of Sirakov et al.(2011), who investigated the profile of ovine PrP gene allele frequencies among eight sheep breeds, reared in Bulgaria.

The present study aimed toestablish the PRNP gene polymorphism based on codons 136, 154 and 171 and its predisposition against the Scrapie prion, among four indigenous sheep breeds reared in Bulgaria.

Materials and Methods

The PrP genotypes were analyzed in sheep and rams from Northeast Bulgarian merino, Karakachan, Copper-Red Shumen and Stara Zagora breed, reared in private farms. Sixty animals from each breed were subjected to investigation, whereas the total number of samples was 240.

Blood samples for analysis was collected aseptically from v. jugulars with disposable needles in plain vacutainers after fixation. Blood was transported in cool bags at 6-7 ºC. DNA was extracted from peripheral blood using QIAamp DNA Mini Kit (Qiagen).

PRNPgenotyping

PrP genotypes were determined by the PCR - RFLP method of Lühken et al. (2004). To distinguish between seven PrP haplotypes based on polymorphisms at codons 136, 154 and 171 we did amplification for two different PCR fragments (197 bp and 196 bp) in separate PCR reactions. The reaction mixture comprised in a volume of 25µl approximately 50 ng of genomic DNA, 20 pmol of each primer, 5mM of each dNTP, 2.0mM (197 bp fragment) or 1.5mM (196 bp fragment) MgCl2 and 0.1U Taq polymerase in 1-fold reaction buffer. After initial denaturation at 95 °C for 5 min, 36 amplification cycles were performed, including denaturation at 95 °C for 30 s, annealing at 56 °C (for both reactions) for 40 s and extension at 72 °C for 1 min, followed by a final 5-min extension at 72 °C. Both fragments were amplified by the same forward primer: 5'-TGTGGCA GGAGCTGCTGCAGCT-3', which is nucleotide 22619 to 22640 of GenBank sequence U67922. The 197 bp fragment was produced with a modified reverse primer:5'-TGCACAAAGTTGTTCTGGTTACTATC-3', which is nucleotide 22791 to 22816 of GenBank sequence U67922, creating an artificial restriction site for BspHI, when the codon for histidine occurs at position 171. The other reverse- primer 5'-GCACAAAGTTGTTCTGGTTACTATAT-3', which is nucleotide 22790 to 22815 of GenBank sequence U67922, amplified the 196 bpfragment with an artificial restriction site for BspDI in the case of the codon for arginine at position 171. In both fragments, the codon for valine at position 136 and for histidine at position 154 form restriction sites for BspHI. The 197 bp fragment was digested with BspHI, while the 196 bp fragment was double digested with BspHI and BspDI simultaneously; samples were incubated for 4 h at 37 °C. Electrophoresis was done in a 3.5% agarose gel (1:1 mixture of low melting agarose and standard agarose) at 7.5V/cm in 1× TBE buffer at 2 °C. The products were visualized after ethidium bromide staining under UV lights.

Statistical analysis

Data analysis was carried out with MEGA4 computer software. Genotypic ($f$ij) and allelic (p□) frequencies were calculated with formulae, used by Gama et al. (2006): $f$ij=nij/N and p□=(2f□□ +∑ $f$ij)/2, where nij is the number of animals with the ij genotype; $f$ij and f□□ are heterozygous and homozygous genotype frequencies,

respectively; p□ is allelic frequency; and N is the total number of animals.

The allelic frequencies within and between investigated breeds were compared by 2x2 contingency tables with χ^2 test set, at 95% confidence interval and critical probability of 0.05.

Result and Discussion

The association between the PrP gene and the Scrapie prion gives us the opportunity for rapid differentiation of susceptible and resistant to the disease individuals. To determine the genetic structure of the investigated sheep populations, we calculated the allelic frequencies of all target alleles and the genotype frequencies formed by them. The allelic frequencies for each investigated breed are shown on table 1. As shown on the table, we detected five PRNP alleles – ARR, ARQ, VRQ, AHQ and ARH, with different prevalence among all breeds.

As we mentioned above, the most resistant to Scrapie allele is ARR. The target PRNP allele, which makes the animals resistant to Scrapie is ARR. This well-known fact motivates breeders to including this allele in their selection programs, as a primary eradication method. The highest ARR allelic frequency was found among the Northwest Bulgarian merino and the Stara Zagora breed (0.43). Their ARR frequencies were significantly higher compared to the Copper-Red Shumen (χ^2 = 11.302, p = 0.0003) and Karakachan (χ^2 = 35.009, p<0,0001).

The wild type allele ARQ was prevalent among all tested breeds with highest frequency among the Karakachan – 0,59. Its rate among the Karakachan breed was significantly higher than that of Stara Zagora and Northwest Bulgarian merino (χ^2 = 12.005, p = 0.0005).

The target allele for the atypical Scrapie - AHQ was detected among all breeds with highest frequency for the Karakachan breed – 0.27. Its frequency among this breed was significantly higher compared with the ones found among the Stara Zagora (χ2 = 14.874, p<0.0017), Copper-Red Shumen (χ^2 = 19.006, p<0.0001) and Northwest Bulgarian merino (χ^2= 39.735, p<0.0001). The Karakachan breed showed significantly higher rates of the AHQ allele than the ARR allele frequency (χ^2= 9.600, p<0.0019).

The rare ARH allele was found only among the Trakia Merino breed, with frequency as low as 7%. Its frequency was significantly lower than that of the ARR allele (χ^2 = 39.735, p<0.0001), ARQ (χ^2 = 33.773, p<0.0001) and VRQ (χ^2 = 3.544, p<0.0598).

The carriers of the VRQ allele are considered as highly susceptible to Scrapie, therefore this allele is the least wanted one from any breeder. The allele was detected in low frequencies among all breeds, with highest values for the Northwest Bulgarian merino – 0.12. The frequency among this breed was significantly higher than the one found among the Karakachan and Copper-Red Shumen breeds (χ^2 = 3.587, p = 0.0582).

The five PrP alleles formed six genotypes ARR/ARR, ARR/ARQ, ARR/ARH, ARQ/ARQ, AHQ/ARQ and ARR/VRQ, each of which belongs to a different Scrapie risk group (Table 2). The genotype frequencies were with different prevalence among the tested breeds.

As we mentioned above, the most resistant to Scrapie and therefore desired genotype is ARR/ARR. This important genotype was detected among all breeds except for the Karakachan breed, where none of the tested

animals was homozygous for the ARR allele. The highest ARR/ARR frequency was observed among the Stara Zagora breed – 0.20, compared with the Copper-Red Shumen (χ^2 = 6.095, p<0.0026) and the Northwest Bulgarian merino (χ^2 = 8.090, p<0.0009).

The ARR/ARQ genotype was observed among all breeds with highest frequency in favor of the Northwest Bulgarian merino – 0.33. The ARR/ARQ genotype frequency among this breed was significantly higher than the one found in Stara Zagora (χ^2 = 6.073, p<0.0039) and Karakachan breed (χ^2 = 14.073, p<0.0011).

The modestly resistant genotype ARR/ARH was detected only among the Northwest Bulgarian merino with frequency of 0.09. Interestingly its frequency among this breed was equal to the one for the ARR/ARR genotype.

The wild type genotype ARQ/ARQ was detected in all breeds with highest frequency in favor of the Copper-Red Shumen – 0.36. Its rates for the Copper-Red Shumen were significantly higher than the ones found among the Northwest Bulgarian merino (χ^2 = 7.261, p<0.0070) and Stara Zagora (χ^2 = 13.225, p<0.0052).

The most susceptible to atypical Scrapie genotype AHQ/ARQ was detected among all breeds with highest rates for the Karakachan breed. This was the most common genotype for this breed with frequency as high as 0.51. The frequency of this genotype for the Karakachan breed was significantly higher than the ones found for the Stara Zagora (χ^2 = 16.539, p<0.0032), Copper-Red Shumen (χ^2 = 21.125, p<0.0014) and Northwest Bulgarian merino (χ^2 = 32.421, p<0.0012). The genetic structure of this breed was additionally complicated by the fact that all investigated rams were carriers of the AHQ allele, which is a predisposition for fast spreading of this allele among the breed.

One of the most susceptible genotypes ARR/VRQ, was spread among all investigated breeds. Its frequency was the highest among the Northwest Bulgarian merino (0.22) followed by the Stara Zagora breed (0.20). The rate of this genotype among the Northwest Bulgarian merino was significantly higher than the one found among the Copper-Red Shumen (χ^2 = 3.457, p<0.0109) and Karakachan breed (χ^2 = 4.157, p<0.055).

The most susceptible genotype VRQ/VRQ was not detected among the four investigated breeds.

Due to the lack of specific treatment and still not well investigated pathogenesis of Scrapie, the primary eradication manner of the disease relies on genotyping and subsequent selection of animals after the resistant (ARR/ARR) genotype. Within the European Community the management of Scrapie is described by the EU Directive 999/2001. Furthermore, the EU Commission in the 2003/100/EU decision lists the conditions and rules for implementation of breeding programs, as part of the management of Scrapie. According to the widely accepted Scrapie eradication plan, small breeders should start the selection with eliminating the highly susceptible VRQ allele and selection of animals heterozygous for the ARR allele. Once the number of ARR allele carriers has reached certain levels, the process could continue with selection of homozygous animals for the ARR allele.

Dawson et al. (2008) define the importance of codons 136, 154 and 171 and their close

relation with the Scrapie infection. The authors divide 15 genotypes into 5 risk groups, each with different susceptibility to the disease. In our investigation we detected six genotypes, where most of the studied animals belonged to groups two and three, which explains the low incidence of Scrapie in Bulgaria.

The role of codon for the amino acid Histidine at codon 154 is still not well researched. According toThorgeirsdottir et al. (1999), the presence of Histidine codon correlates with risk of the disease, but they classify this genotype into group 3. Lühken et al. (2004) reported that this particular genotype is responsible for high susceptibility among some German breeds. These differences could be explained with the different breeds that authors have studied and the impossibility of genotyping all Scrapie positive sheep. Based on these divergent outcomes, we could classify the AHQ allele as high risk allele. It's frequency among the Bulgarian breeds was relatively low, except for the Karakachan breed where its value was as high as 27%. We should point out that the frequency of the moderately susceptible genotype AHQ/ARQ among this breed was 51%, which unambiguously shows the lack of appropriate breeding program in our country.

The wild allele ARQ is considered susceptible to the disease especially in herds with low frequency of the VRQ allele (Tranulis, 2002). We should mention that the VRQ allele is target for some Scrapie strains, while for others the target allele is ARQ (Belt et al., 1995). Among the breeds investigated in our study, the rate of the ARQ allele was highest for the Karakachan breed. Having in mind the high frequency of the AHQ allele among this breed, we could definitely see a risk of the disease. The most resistant allele ARR was highest among the Stara Zagora and Northwest Bulgarian merino (43%), which in our opinion is a result of arbitrary selection. Even though the allele frequency was high, the homozygous animals for the ARR allele were about 20% for the Stara Zagora and only 9% for the Northwest Bulgarian merino. Both breeds had individuals carrying the most susceptible allele VRQ. Taking into account the obtained results among the investigated breeds we could recommend the three stages breeding method of Windig et al.(2004):

1. Mild selection – using homozygous and heterozygous for the ARR allele rams and ewes from any genotype, except for the VRQ allele carriers.
2. Moderate selection – using predominantly ARR/ARR rams.
3. Severe selection – using only ARR/ARR rams.

The relatively high frequency of the ARR allele among the Northwest Bulgarian merino and Stara Zagora breed, allows the process to start from the second stage. The ARR allele frequency for the other two local breeds is relatively low, so moderate and severe selection could result in high inbreeding levels. For these breeds we recommend mild selection with prior elimination of all VRQ carriers and ARQ homozygous rams. Once the number of ARR carriers reach certain level, the process could continue with moderate and severe selection (Molina et al., 2006).

Sirakov et al. (2011) investigated eight indigenous breeds – Copper-Red Shumen, Bulgarian Dairy Sinthetic Population, Pleven Blackhead breed, StaraPlaninaTzigay, Replyan sheep, Karakachan sheep, Duben sheep and Stara Zagora, reared in Bulgaria. Authors detected some of the most susceptible genotypes –

ARR/VRQ and ARQ/VRQ, which indicates the necessity of a breeding program. The results for the Stara Zagora breed are almost identical to ours, with low rates of the ARR/ARR genotype and presence of the VRQ allele. The results for the rest of the breeds show carriers of one of the most susceptible alleles – VRQ. Combining the results obtained by Sirakov et al. (2011) with our data, we could confirm the highly divergent PRNP polymorphism among the Bulgarian local breeds, which indicates the need of immediate Scrapie eradication program in our country.

Authors from different countries have investigated the PRNP polymorphisms, with aim to clarify the risk of Scrapie among different breeds. Acín et al.(2004) investigated the polymorphism of the three codons among the Rasa Aragonesa, Ojinegra, Cartera, Maellana, Roya Bilbilitana, Ansotana and ChurraTensina breeds, reared in Spain. The reported results indicate very low frequency of the ARR/ARR genotype for all studied breeds and relatively high rates (20%) of the susceptible ARQ/VRQ genotype among the Maellana breed. Authors seek a huge risk for this breed and recommend immediate genotyping and selection after the resistant genotype.

The complicated nature of the Scrapie prion and the absence of special treatment for the disease, do not give us any other choice, but genotyping and selection of animals with the resistant genotype ARR/ARR. Unfortunately this genotype is not widely spread among any breed, so we need implementation of specific breeding program for each breed of interest. The PRNP genotypes detected among the Bulgarian local breeds, carry moderate risk for our sheep industry, but the presence of the VRQ allele undoubtedly indicates the lack of appropriate Scrapie resistant breeding program in our country. The obtained results help for better understanding of the disease and would be useful for designing a successful Scrapie eradication program.

Table.1 PRNP gene allelic frequencies among four indigenous sheep breeds, reared in Bulgaria.

Breed/allele	**N**	**PRNP^{ARR}**	**PRNP^{ARQ}**	**PRNP^{AHQ}**	**PRNP^{ARH}**	**PRNP^{VRQ}**
Northeast Bulgarian merino	60	0,43	0,35	0,03	0,07	0,12
Karakachan	60	0,06	0,59	0,27	0,00	0,08
Copper-Red Shumen	60	0,29	0,56	0,07	0,00	0,08
StaraZagora	60	0,43	0,35	0,11	0,00	0,11

Table.2 PRNP genotype frequencies among four indigenous sheep breeds, reared in Bulgaria. Risk groups according to (DEFRA, 2001).

Scrapierisk group	**n**	**1**	**2**		**3**		**4**
Breed/genotype		ARR/ARR	ARR/ARQ	ARR/ARH	ARQ/ARQ	AHQ/ARQ	ARR/VRQ
Northeast Bulgarian merino	60	0,09	0,33	0,09	0,20	0,07	0,22
Karakachan	60	0,00	0,11	0,00	0,31	0,51	0,07
Copper-Red Shumen	60	0,12	0,28	0,00	0,36	0,12	0,12
StaraZagora	60	0,20	0,23	0,00	0,15	0,22	0,20

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