

Analysis of the ORF region of the prion protein gene in the Botosani Karakul sheep breed from Romania

Steliana Elvira Maria KEVORKIAN*, Mihaela ZĂULEȚ, Maria Adina MANEA,

Sergiu Emil GEORGESCU, Marieta COSTACHE

University of Bucharest, Department of Biochemistry and Molecular Biology, Spl. Independentei 91-95,
Bucharest - ROMANIA

Received: 30.09.2009

Abstract: Ovine scrapie is considered a neurodegenerative disease characterized by the accumulation of an abnormal, protease-resistant isoform of PrP. The polymorphisms at codons 136, 154, and 171 are associated with susceptibility to scrapie. In order to detect the existing polymorphisms at the 3 codons we used the real-time PCR technique. The obtained genotypes were confirmed by PCR amplification and sequencing of a 402 bp fragment of exon 3 of the prion gene. Known polymorphisms were detected at codons 136, 154, and 171 and 5 different genotypes were determined. The results showed that all animals were homozygous for A at codon 136 and homozygous for R at codon 154 and 3 out of 4 previously identified polymorphisms at residue 171 were detected (R, H, and Q). Two new polymorphisms were detected at codon 189 and 199, as well as 2 other reported polymorphisms at codons 141 and 143. The frequency of high risk ARQ/ARQ genotype was about 47%. The aim of this study was to determine some preliminary information on the PrP gene-associated susceptibility in scrapie in the Botosani Karakul sheep breed.

Key words: Sheep, scrapie, PRNP, real-time PCR, sequencing

Introduction

The prions represent infectious particles that cause neurodegenerative diseases characterized by degeneracy of the central nervous system. The process that causes the disease is represented by the conversion of the normal protein (PrP^c), synthesized in all mammals' brain in an abnormal, mutant one (PrP^{Sc}). Scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer, and Creutzfeld-Jacob disease (CJD) in humans are all transmissible prion diseases called transmissible spongiform encephalopathies (TSEs).

In sheep the PrP gene is located on chromosome 13 and has 3 exons (1). Both ovine and caprine genes exhibit the alternative polyadenylation of their PrP messenger RNA (2).

Although polymorphisms appear in the entire sheep PrP gene, only those located in the protein coding region have been associated with TSE susceptibility. Polymorphism of the ovine PrP gene associated with natural or induced scrapie was firstly detected at the beginning of 1990. The first detected ovine polymorphism associated with scrapie was variant Gln171Arg (Q₁₇₁R) (3), followed by the association

* E-mail: stelyana2004@yahoo.com

between Ala136Val(A₁₃₆V) polymorphism and the period of incubation after the animal was exposed to experimental scrapie. Val₁₃₆, Arg₁₅₄, Gln₁₇₁, and His₁₇₁ (V₁₃₆, R₁₅₄, Q₁₇₁, H₁₇₁) represent the alleles linked to susceptibility to scrapie.

The Karakul breed may be one of the oldest breeds of domesticated sheep. It originated in Central Asia. The Botosani Karakul breed was formed in Botosani county in Romania after the inbreeding of different Karakul sheep from Germany and Austria with Tsurcana rams from Romania and includes about 750,000 sheep. This unique breed is mainly raised for pelts and therefore the lambs are sacrificed at a maximum of 7 days after birth. So far no studies have been carried out on the Botosani Karakul breed regarding the incidence of scrapie.

In Romania in 2009 out of 1100 scrapie likely cases from several different sheep breeds collected from 7 different counties (Bacau, Bihor, Botosani, Braila, Bucharest, Calarasi, and Caras-Severin), only 3 cases were detected.

Materials and methods

Sampling and DNA extraction

Brain tissue samples were collected from 100 randomly chosen animals belonging to 2 flocks of Botosani Karakul sheep. Genomic DNA was isolated using a High Pure PCR Template Preparation Kit from Roche Diagnostics according to the manufacturer's instructions.

Genotype Real Time PCR with melting curve analysis

The aim of this study was to determine some preliminary information on the PrP gene-associated susceptibility in scrapie in Botosani Karakul sheep breed, considering that this breed is the main pelt breed in Romania.

Genotype analysis was performed using the real-time PCR system LightCycler 2.0 (Roche). The gene mutations in the codons 136, 154 and 171 were checked using the dual colour LightCycler Scrapie Susceptibility Mutation Kit (TIB MOLBIOL, Germany). The kit was used in conjunction with the LightCycler FastStart DNA Master Hybridization Probes (Roche Applied Science). Performing this multiplex PCR and melting curve analysis we were able to identify the allelic variants in a single run. In order to ensure the reproducibility of our results all samples were run in duplicate. The used parameters for the real time PCR reactions are described in Table 1.

In order to obtain the reaction mix used for real-time PCR reactions we combined the components described in Table 2.

PCR conditions and reactions

In order to confirm the real-time results we amplified by PCR and sequenced a region containing the 3 codons.

The amplification reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) in a 25 µL final volume reaction and consisted of PCR

Table 1. The program used to obtain real-time PCR reactions.

Program	No of cycles	Temperature	Time
Pre-Incubation	1	95 °C	8 min
		95 °C	10 sec
Amplification	45	60 °C	10 sec
		72 °C	15 sec
Melting curve	1	95 °C	2 min
		45	60 sec
		45-75/raise by 0,2 °C/sec	1 sec
Cooling	1	40 °C	30 sec

Table 2. Components of real-time PCR reaction.

Components	Volumes (μl)
MgCl ₂	0,8
Mix of primers and probes	4
Mix of polymerase, dNTP, buffer	2
Deionized water	11,2
DNA	2
Total Volume	20

Buffer, MgCl₂, dNTPs, DNA template (50 ng), 0.5 units of AmpliTaq Gold DNA Polymerase, 20 μM of each primer (F-GGTCAAGGTGGTAGCCACAGTC AGTGGAAC and R-AGCCTGGGATTCTCTCTG GTACTGGGTGAT), and nuclease free water. PCR amplifications were performed using a program with 40 cycles. Denaturation was performed at 95 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 1 min. The first denaturation step was of 10 min at 95 °C and the final extension was of 10 min at 72 °C. The primers used in this study were designed with the Primer 3 program based on the existing ovine prion protein gene sequence from GenBank DQ408530. The amplified fragment was of 402 bp and covered the sequence between position 280 and 681 and encoded 134 amino acids.

Sequencing and sequence analysis

To confirm the obtained genotypes we sequenced the products. The PCR products were purified using the Wizard System Kit (Promega) according to the manufacturer's instructions. The purified products were mixed with an ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit and were analyzed on an ABI Prism 3130 Genetic Analyzer. The sequencing step was done in both directions using the same primers described for the PCR reaction. The sequences were processed using DNA Sequencing Analysis 5.1 Software (Applied Biosystems) and the nucleotide sequences were aligned using BioEdit software.

Results

The LightCycler® FastStart DNA MasterPLUS HybProbe kit used to obtain the real-time PCR reactions is a ready-to-use reaction mix designed

specifically for the HybProbe probes detection format based on the assumption of mutual exclusivity using the LightCycler® Carousel-Based System. It is used to perform hot start PCR in glass capillaries, which has been shown to significantly improve the specificity and sensitivity of PCR (4) by minimizing the formation of non-specific amplification products at the beginning of the reaction.

Using the real-time PCR technique we identified the following genotypes: ARQ/ARQ, ARH/ARQ, ARR/ARH, ARR/ARQ, and ARR/ARR. The most frequent allele was ARQ.

The influence of polymorphism of codon 154 in susceptibility to scrapie is lower compared with codons 136 and 171. Still it is known that animals carrying the H allele are less susceptible to classical scrapie but are more sensitive to BSE and atypical scrapie. In this study we found only the 154RR genotype and no 154HH genotype.

In codon 171 we observed 3 alleles and 5 different genotypes (Figure 1).

The observed genotypes of the PrP gene in the Botosani Karakul breed and their frequencies are summarized in Table 3.

The different genotypes obtained in real-time PCR were sequenced in order to confirm the results. We sequenced 20 animals from each risk group. Using the ClustalW application from the BioEdit program we aligned the ARQ/ARQ and ARR/ARR obtained genotypes with the sequence for the prion protein gene from GenBank.

Four additional polymorphisms were detected by sequencing at codons 141, 143, 189, and 199 (Figure 2). Two of these polymorphisms have been described before, while the other 2 represent undescribed polymorphisms.

Discussion

Scrapie resistant breeding programs are based on the prion protein (PrP) genotype. In this study we identified 5 different alleles at codons 136, 154, and 171, and the variation appeared only in codon 171.

The data in the literature have shown that sheep presenting the ARR allele are the most resistant to scrapie both in homozygosis and heterozygosis,

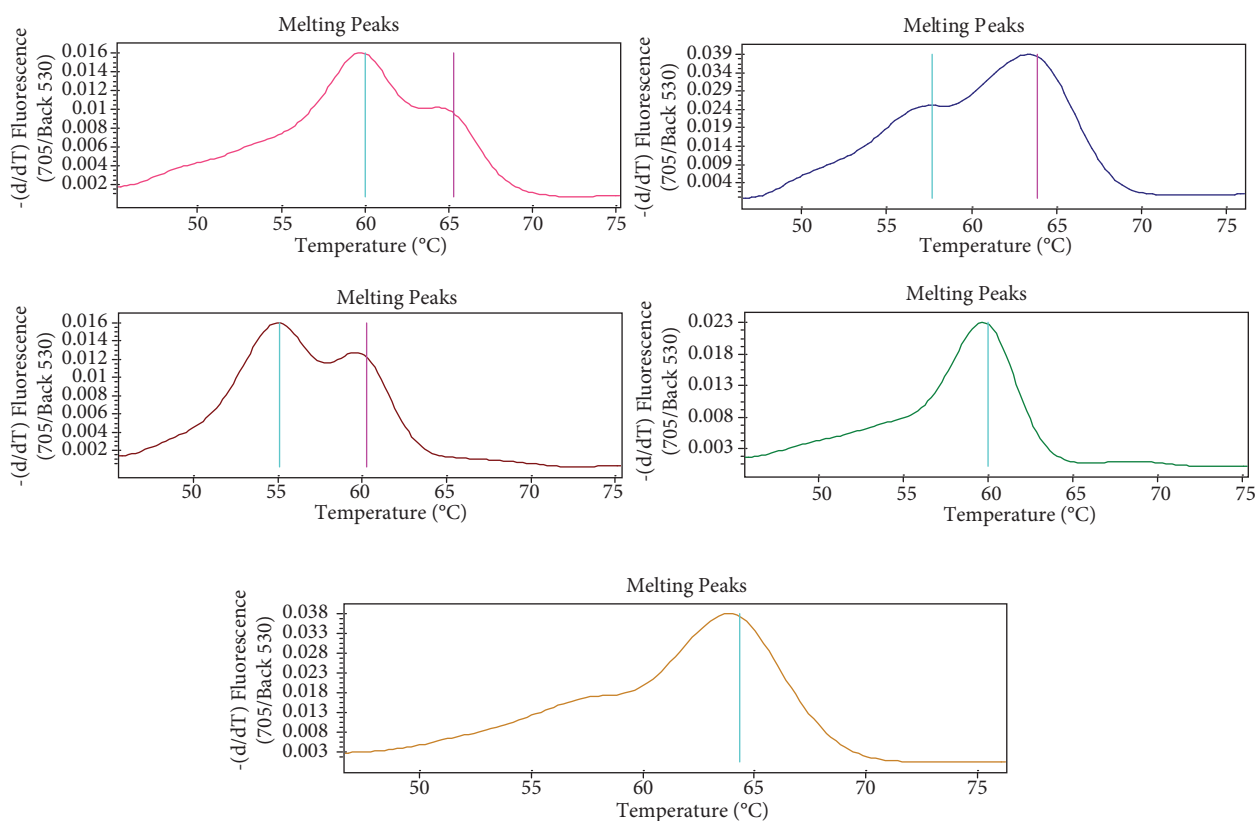


Figure 1. Melting curves obtained for codon 171 and the obtained genotypes.

Table 3. Genotype frequencies of the PrP gene in the Botosani Karakul breed.

Risk group	Number of samples	PrP genotype	Frequencies %
1	31	ARR/ARR	31
2	9	ARR/ARH	9
2	12	ARR/ARQ	12
3	47	ARQ/ARQ	47
3	1	ARH/ARQ	1

whereas ARH/ARH and ARH/ARQ were the least resistant genotypes to scrapie. The ARQ/ARQ is a highly susceptible genotype for scrapie (5,6).

In the Botosani Karakul breed the ARR/ARR genotype was observed in about 31% of animals. About 12% of the animals carried the ARR/ARQ genotype and 9% carried the ARR/ARH genotype. Even if the animals belonging to risk class 2 are unlikely to develop scrapie, their use in breeding should still be strictly considered (7).

Polymorphism at codon 136 is associated with susceptibility of various scrapie strains. In the present study we observed only the presence of alanine and we identified no 136VV genotype. Sheep with arginine at codon 171 rarely develop natural scrapie and generally show long incubation periods after experimental challenge (5,8,9).

Apart from the polymorphisms detected at codons 136, 154, and 171, we determined 4 other polymorphisms. The His-Arg substitution at codon

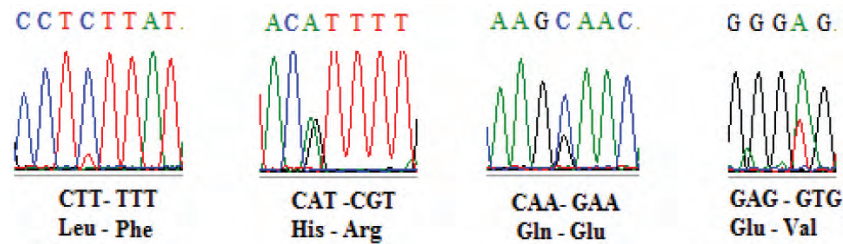


Figure 2. Four additional polymorphisms detected in the analyzed region.

143 was also detected in the Ujumqin breed from Mongolia (10) and Dorset and Suffolk breeds from the USA (11). The Leu-Phe substitution in codon 141 was reported also in the Flemish and Swifter breed (12) and also in Dorset and Suffolk breeds from the USA (11). We identified 2 new undescribed polymorphism at codon 189 (Gln * Glu) and at codon 199 (Glu*Val). In all these polymorphisms the individuals were heterozygous. It is very interesting to see in future studies if these additional

polymorphisms in ovine PrP gene are associated with susceptibility or resistance to scrapie.

This study represents preliminary research on genetic susceptibility to scrapie in the Botosani Karakul breed. The economical importance of this breed is represented by the obtained pelts. Knowing the PrP genotype of sheep and rams is a valuable tool to assess the susceptibility of animals to develop scrapie and therefore to obtain only highly resistant animals to scrapie.

References

1. Castiglioni, B., Comincini, S., Drisaldi, B., Motta, T., Marchitelli, C., Valentini, A., Pagnacco, G., Leone, P. & Ferretti, L.: Physical mapping of the prion genes in cattle, sheep and man by fluorescence in situ hybridization. *Proceedings of the 6th World Congress on Genetics Applied to Livestock Production*, 1998; 27: 343-346.
2. Goldmann, W., O'Neill, G., Cheung, F., Charleson, F., Ford, P., Hunter, N.: PrP (prion) gene expression in sheep may be modulated by alternative polyadenylation of its messenger RNA. *J. Gen. Virol.*, 1999; 80: 2275-2283.
3. Goldmann, W., Hunter, N., Foster, J.D., Salbaum, J.M., Beyreuther, K., Hope, J.: Two alleles of a neural protein gene linked to scrapie in sheep. *Proc. Natl. Acad. Sci. USA*, 1990; 87: 2476-2480.
4. Roche Diagnostics, PCR Manual, 2nd edition, 1999; 52-58.
5. Hunter, N., Foster, J.D., Goldmann, W., Stear, M.J., Hope, J., Bostock, C.: Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Arch. Virol.*, 1996; 141: 809-824.
6. Moum, T., Olsaker, I., Hopp, P., Moldal, T., Valheim, M., Benestad, S.L.: Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases. *J. Gen. Virol.*, 2005; 86: 231-235.
7. Dawson, M., Hoinville, L.J., Hosie, B.D., Hunter, N.: Guidance on the use of PrP genotyping as an aid to the control of clinical scrapie. *Scrapie Information Group. Vet. Rec.*, 1998; 142: 623-625.
8. Belt, P.B., Muileman, I.H., Schreuder, B.E., Bos-de Ruijter, J., Gielkens, A.L., Smits, M.A.: Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *Journal of General Virology*, 1995; 76, 509-17.
9. Goldmann, W., Martin, T., Foster, J., Hughes, S., Smith, G., Hughes K., Dawson, M., Hunter, N.: Novel polymorphisms in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period. *Journal of General Virology*, 1996; 77: 2885-91.
10. Wang, Y, Qin, Z., Qiao, J., Zhao, D.: Polymorphisms of the prion protein gene in sheep of Inner Mongolia, *China Virus Genes.*, 2008; 37: 128-130.
11. Desilva, U., Guo, X., Kupfer, D.M., Fernando, S.C., Pillai, A.T.V., Najar, F.Z., So, S., Fitch, G.Q., Roe, B.A.: Allelic variants of ovine prion protein gene (PRNP) in Oklahoma sheep, *Cytogenet. Genome Res.*, 2003; 102: 89-94.
12. Bossers, A., Schreuder B.E.C., Muileman, I.H., Belt, P., Smits, M.A.: PrP genotype contributes to determining survival times of sheep with natural scrapie. *J. of Virology*, 1996; 77: 2669-2673.