PrP GENE POLYMORPHISM IN SHEEP BREEDS IN SLOVAKIA AND SUSCEPTIBILITY TO SCRAPIE

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Received November 2, 2005; accepted October 24, 2006

Summary. – We analyzed the prion protein (PrP) genotype based on the codons 136, 154 and 171 and assigned to five risk groups (R1-R5) in healthy and scrapie-affected sheep in Slovakia. In healthy (asymptomatic) population, 119 Merino, 106 Improved Valachian, 117 Tsigai, and 48 Suffolk breeds were tested. Among the asymptomatic sheep, the low-risk genotypes R1 and R2 were most abundant in Suffolk (94%) and Merino (84%) breeds, followed by Tsigai (58%) and Improved Valachian (40%) breeds. The medium-risk group R3 was most frequent in Improved Valachian (31%) breed, followed by Tsigai (21%), Merino (10%), and Suffolk (6%) breeds. The occurrence of high-risk groups R4 and R5 was none in Suffolk breed, followed by Merino (6%), Tsigai (21%), and Improved Valachian (30%) breeds. Since 2003, altogether 48 cases of scrapie have been confirmed in Tsigai (38), Merino (4), Improved Valachian (2), Improved Valachian x Tsigai (3), and Suffolk (1) breeds. Among sheep with scrapie, Merino breed belonged to the medium-risk group R3. The majority of scrapie-affected Tsigai sheep were classified into high-risk R5 (50%) and medium-risk R3 (42%) groups. We showed an association of scrapie with medium- and high-risk groups of PrP genotype in Slovakia. In particular, the glutamine at position 171 appears to be of major importance for the susceptibility to scrapie.

Key words: scrapie; PrP; polymorphism; prion; sheep breeds

Introduction

Scrapie is a neurodegenerative disease of the transmissible spongiform encephalopathy (TSE) group affecting adult sheep and goats. The disease is associated with conversion of a normal host-encoded cellular PrP (PrP^{C}) into a misfolded scrapie conformer (PrP^{Sc}). Accumulation of protease-resistant PrP^{Sc} in CNS is a hallmark of scrapie and other TSE and is used as a diagnostic marker (Novak *et al*., 2000).

In the ovine PrP gene, most of polymorphisms occur between the codons 98 and 234 (Hunter *et al*., 1989; Baylis and Goldmann, 2004). Among them, the nucleotide variations in the codons 136, 154 and 171 are predominantly linked to scrapie susceptibility (Hunter *et al*., 1989). These codons determine the polymorphism of amino acids at the corresponding positions in PrP protein. On the basis of the polymorphism at these three positions, sheep are classified into five risk groups, R1 to R5 (www.defra.gov.uk) (Baylis and Goldmann, 2004).

For detection of the PrP-gene polymorphism different methods have been used, in particular DNA sequencing, restriction fragment length polymorphism analysis, allele-specific amplification, and denaturing gradient gel electrophoresis (DGGE) coupled with the DNA sequencing.

A policy of sheep breeding for scrapie resistance and basic culling strategies based on PrP genotyping has been realized in European Union since 2003. However, in many countries including Slovakia, the relationship between scrapie

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Abbreviations: DGGE = denaturing gradient gel electrophoresis; PrP = prion protein; TSE = transmissible spongiform encephalopathy

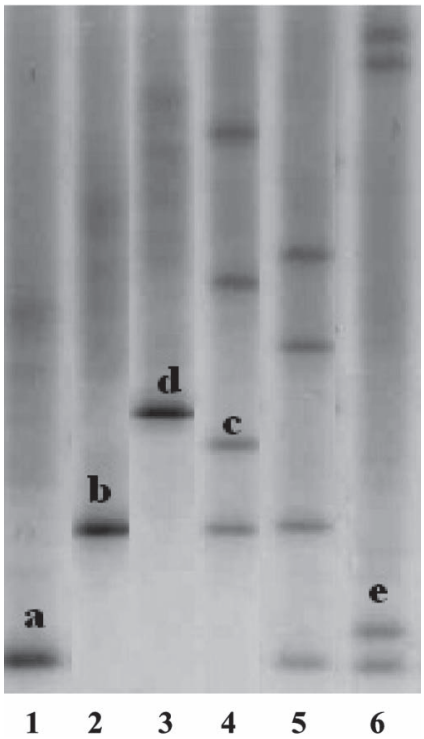

Fig. 1

Representative DGGE profiles of PrP genotypes

DGGE profiles of 6 different PrP genotypes. The genotypes: ARR/ARR (lane 1), ARQ/ARQ (lane 2), VRQ/VRQ (lane 3), ARQ/ARH (lane 4), ARR/ARQ (lane 5), and ARR/ARQ with polymorphism of the codon 146 (N146S) (lane 6). The alleles: ARR (a), ARQ (b), ARH (c), VRQ (d), and ARQ with a mutation in codon 146 (N146S) (e).

susceptibility and PrP genotypes has not been studied yet. In Slovakia, three major sheep breeds are reared, namely Improved Valachian in highlands, Merino in lowlands, and Tsigai in middle altitudes. These breeds account for about 87% of sheep population in the country. The first confirmed ovine scrapie in the country occurred in 2003 (Matúšková *et al*., 2003). Since then, 48 scrapie-affected sheep (38 Tsigai, 4 Merino, 2 Improved Valachian, 3 Improved Valachian x Tsigai, and 1 Suffolk breed) have been detected. Therefore the aim of this study was to determine the incidence of individual PrP genotypes in various breeds in asymptomatic and in scrapie-affected sheep in Slovakia.

Materials and Methods

Animals. A total of 390 asymptomatic sheep of 4 different breeds (106 Improved Valachian, 119 Merino, 117 Tsigai, and 48 Suffolk) and 48 scrapie-affected sheep (2 Improved Valachian, 4 Merino, 38 Tsigai, 1 Suffolk, and 3 Improved Valachian x Tsigai) were included in the study. Leukocytes isolated from EDTA-treated blood samples were used for extraction of genomic DNA. The presence of protease-resistant PrP in brain specimens was examined by rapid tests Enfer TSE (Enfer Scientific Limited, UK) and Prionics Check Western (Prionics AG, Switzerland) and confirmed immunohistochemically using a monoclonal antibody 6H4.

PCR, DGGE and sequencing of PrP gene from asymptomatic sheep. A 176 bp fragment of PrP gene (nt 450–626) from asymptomatic sheep was amplified by PCR from genomic DNA (Sambrook *et al.*, 1989) using the primers pTK-F1 (5'-GGCCTTGGTGGCTACATGCTG-3', forward) and pTK-R1 (5'-CGCCCGCCGCGCCCCGCGCCCG CCCGCCGCCCCCGCCCGTTTTTTATGTTGACACAG TCATGCAC-3', reverse). The PCR products were denaturated at 94°C for 15 mins, renaturated at 50°C for 10 mins, and subjected to DGGE (Belt *et al*., 1995). The electrophoresis ran in 6% polyacrylamide gel with linear 20–65% gradient of denaturants (urea and formamide) in 0.5x TAE buffer (40 mmol/l Tris-acetate and 1 mmol/l EDTA) at 58°C in a DGGE unit (Ingeny, the Netherlands). Gels were silver-stained and the resulting DGGE profiles were recorded. From DNA samples corresponding to each typical DGGE profile a 451 bp fragment of PrP gene (nt 358–809) was amplified by PCR using the primers pTK-F2 (5'-GTGGTAGCCTCAGTCAGTGGAACA-3', forward) and pTK-R2 (5'-GAGGAGGATCACAG GAGGGGAA-3', reverse). The PCR products were sequenced in an ABI Prism™ 377 Perkin Elmer Sequencer using the Big Dye Terminator Kit (Applied Biosystems). The sequences were analyzed using the Sequence Navigator Program (Perkin Elmer) and Multalin Program (http://prodes.toulouse.inra.fr/multalin/multalin.html), respectively, and compared with known sequences of sheep PrP gene available in GenBank. The sequences of novel alleles were deposited at GenBank under Acc. Nos. AY822665-AY822672.

PCR and sequencing of PrP gene from scrapie-affected sheep. Complete coding region of PrP gene was amplified by PCR from genomic DNA (Matúšková *et al*., 2003). The PCR products of 770 bp were sequenced and analyzed as above.

Statistical analysis. The two-sided Fisher's exact test and the Prism 3.03 Program Tool (GraphPad, USA) were employed.

Results and Discussion

A correlation of the susceptibility of sheep to scrapie with the PrP genotype is well known. In particular, the polymorphisms at the codons (amino acids) 136, 154 and 171 play the most significant role in this respect (Hunter *et al*., 1996). Therefore the genotype analysis of PrP gene, in particular the polymorphisms at the codons mentioned

above in asymptomatic and scrapie-affected sheep in Slovakia was performed. For detection of the PrP gene polymorphism DGGE was chosen, as it proved to be a rapid and convenient technique for analyzing a large number of samples.

In 390 asymtomatic sheep, 26 different DGGE profiles were found. Some of them are presented in Fig. 1. DNA samples corresponding to individual DGGE profiles were subjected to sequencing of complete PrP gene. In this way, individual DGGE profiles could be assigned to respective alleles and genotypes (amino acids at positions 136, 154 and 171) without sequencing and not all DNA samples had to be sequenced.

A total of 5 alleles (ARR, ARH, AHQ, ARQ, and VRQ) of PrP gene were identified (Table 1). Combination of involved PrP alleles resulted in 11 PrP genotypes representig all five risk groups R1-R5. According to their distribution in the risk groups, ARR is considered a no-risk allele, ARH, AHQ, and ARQ low- to medium-risk alleles, while VRQ a high-risk allele (Hunter, 1997). The no-risk ARR allele was predominant in Suffolk (66%) and Merino (63%) breeds, but was relatively high also in Tsigai (43%) and Improved Valachian (32%) breeds. ARH was rare and found only in Merino and Suffolk breeds with frequencies of 3% and 1%, respectively (Table 2). The incidence of AHQ was highest in Improved Valachian breed (14%), but in other breeds was marginal (Merino and Suffolk) or none (Tsigai).

In Improved Valachian breed, ARR/ARQ (R2) was the dominant genotype, observed in 19.8%–20 % of sheep. In general, the occurrence of low- to medium-risk genotypes (R1-R3) was 71%, while that of high-risk genotypes (R4 and R5) was 29%. In Merino breed, the low-risk ARR/ARR genotype (R1) was dominant (42%), while in Tsigai and Suffolk breeds, the low-risk ARR/ARQ genotype (R2) was most frequent (Table 1.)

In Merino, Tsigai and Suffolk breeds, the occurrence of low-risk genotypes (R1 and R2) was 84%, 58% and 94%, respectively, and that of medium-risk genotype (R3) was 10%, 21% and 6%, respectively. The occurrence of high-risk genotypes (R4 and R5) in the same breeds was 6%, 21%, and 0%, respectively. High-risk genotypes (R4 and R5) were absent in Suffolk breed, which determines a relative resistance of this breed to scrapie. Drogemuller *et al.* (2001) have not found the high-risk genotype VRQ/VRQ (R5) in Suffolk breed either.

In asymptomatic sheep, the occurrence of ARQ was highest in Tsigai (45%) and Improved Valachian (39%) breeds, while it was 28% both in Merino and Suffolk breeds (Table 2). ARQ is considered to determine the high-risk genotype of Suffolk breed, as VRQ did not occur there. In Improved Valachian, Merino and Tsigai breeds, the high-risk VRQ allele was at comparable level (10–15%). AHQ has been found frequently in scrapie-affected German Merinoland breed (Luhken *et al*., 2004).

Most of the ovine di- or polymorphisms described to date occurred between the codons 98 and 234 (Baylis and Goldmann, 2004). This study identified known polymorphisms of the type A136V, H143R, R154H, R171H,

Table 1. Incidence of various PrP genotypes and risk groups in sheep breeds

Risk groups	Genotypes	Incidence (%)			
		Improved Valachian (n = 106)	Merino (n = 119)	Tsigai (n = 117)	Suffolk (n = 48)
R1	ARR/ARR	9	42	15	40
R2	ARR/AHQ	10	2	0	4
	ARR/ARQ	20	40	43	48
	ARH/ARR	0	1	0	2
	Total	30	43	43	54
R3	ARQ/AHQ	14	1	0	2
	ARQ/ARQ	16	8	21	0
	ARQ/ARH	0	2	0	4
	AHQ/AHQ	1	0	0	0
	Total	31	11	21	6
R4	ARR/VRQ	15	4	13	0
R5	ARQ/VRQ	13	2	7	0
	VRQ/VRQ	1	0	2	0
	Total	14	2	9	0

Table 2. Incidence of individual PrP alleles in various sheep breeds

Alleles	Incidence (%)			
	Valachian	Merino	Tsigai	Suffolk
ARR	32	60	43	66
AHQ	14	1	0	3
ARH	0	1	0	3
ARQ	39	28	45	28
VRQ	15	10	12	0

Table 3. Distribution of individual genotypes, risk groups and polymorphisms at codon 171

Genotypes	Risk groups	Polymorphisms at codon 171	
		Type	No. (%) of sheep
ARR/ARR	R1	RR_{171}	1 (2)
ARQ/ARQ	R3	QQ_{171}	42 (88)
VRQ/VRQ	R5		
ARQ/VRQ	R5		
ARQ/AHQ	R3		
ARR/ARQ	R2	RQ_{171}	3 (6)
ARH/ARQ	R3	HQ_{171}	2 (4)
ARH/VRQ	R5		

A total of 48 scrapie-affected sheep were examined.

Table 4. Distribution of individual PrP genotypes and risk groups in asymptomatic and scrapie-affected Tsigai sheep

Risk groups	Genotypes	Asymptomatic sheep (n = 117)	Scrapie-affected sheep (n = 38)	Differences between respective pairs of risk groups
R1	ARR/ARR	18	0	Significant (P<0.01)
R2	ARR/ARQ	50	3	Significant (P<0.001)
R3	ARQ/ARQ ARH/ARQ ARQ/AHQ	24 0 0	17 1 1	Significant (P<0.001)
R4	ARR/VRQ	15	0	Significant (P<0.005)
R5	ARQ/VRQ VRQ/VRQ ARH/VRQ	8 2 0	13 2 1	Significant (P<0.0001)

Table 5. Incidence of individual PrP alleles in asymptomatic and scrapie-affected Tsigai sheep

Sheep	Incidence (%)				
	ARQ	VRQ	ARH	ARR	AHQ
Asymptomatic (n = 117)	45%	12%	0%	43%	0%
Scrapie-affected (n = 38)	68%	24%	3%	4%	1%

R171Q, and N176K. Moreover, two novel polymorphisms, namely N146S and P168L were observed (Fig. 1). The presence of polymorphisms at the codon 146 was recorded in one Improved Valachian and one Suffolk sheep. In six Improved Valachian sheep, polymorphism of the codon 168 was noticed. Recently, dimorphism of the codon 168 was identified in goats (Billinis *et al*., 2002).

PrP genotyping in 48 scrapie-affected sheep demonstrated that, except for 4 animals, all positive cases had genotypes of medium-risk (R3) or high-risk group (R4, R5) groups (Table 3). Among the scrapie-affected sheep, 42 (88%) were homozygous with QQ_{171}, 1 was homozygous with RR_{171}, 2 were heterozygous with HQ_{171}, and 3 were heterozygous with RQ_{171}. In contrast, among 390 asymptomatic sheep, only 17% were homozygous with QQ_{171}, a risk factor for scrapie; this difference was highly significant (P<0.0001).

Because all scrapie-positive Tsigai sheep (38) originated from a single flock, we compared asymptomatic and scrapie-affected animals of this breed for the distribution of individual PrP genotypes (Table 4). The results showed that the frequency of high-risk PrP genotype R5 in sheep with scrapie was significantly higher than that in asymptomatic sheep (P<0.0001). In contrast, the low-risk R1 and R2 genotypes had a significant protective effect against scrapie (P<0.01 and P<0.001, respectively). We also compared asymptomatic and scrapie-affected sheep of the same breed for the frequencies of individual PrP alleles (Table 5). The results showed that the frequencies of the high-risk alleles ARQ and VRQ in scrapie-affected population were substantially higher than those in asymptomatic one. These findings are consistent with a conclusion that the glutamine 171 has a significant effect on scrapie susceptibility.

Our data support the linkage between the medium and high-risk PrP genotype groups and the vulnerability to scrapie. A higher occurrence of the high-risk R4 and R5 groups in asymptomatic sheep of Improved Valachian (total 29%) and Tsigai (total 21%) breeds indicates their potentially increased susceptibility to scrapie. Elimination of sheep with high-risk PrP genotypes is an effective tool for maintaining healthy breeds. Results of our study highlighted the relevance of broad-range PrP genotyping in sheep for elimination of carriers of high-risk PrP alleles and hence for increase of overall resistance of sheep population to scrapie (Tkacikova *et al*., 2003).

Acknowledgements. This work was supported in part by the grants Nos. 2/2328/05, 2/4125/26 and 2/5102/26 from the Scientific Grant Agency of Ministry of Education of Slovak Republic AND Slovak Academy of Sciences, and by the State Program of Research and Development No. SP51 028 08 00/028 008 03 "Genomics of transmissible diseases for healthier population of human and animals".

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