FIRST CONFIRMED SHEEP SCRAPIE WITH $A^{136}R^{154}Q^{171}$ GENOTYPE IN SLOVAKIA

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Summary. – The first confirmed evidence of scrapie in Slovakia was demonstrated in one sheep of the autochthonous Merino breed from the southeastern part of the country. The reported scrapie was diagnosed during compulsory transmissible spongiform encephalitis (TSE) screening of sheep over 9 months of age assigned for consumption. The positive ewe was 5-year-old, which did not show any clinical signs of scrapie. The presence of the proteinase-resistant prion protein (PrP) in brain was proved independently by two laboratories using two different immunochemical screening systems, namely the Prionics Check (Western blot analysis) and Enfer TSE enzyme-linked immunosorbent assay (ELISA). In addition, the genotyping analysis of PrP gene demonstrated the presence of PrP genotype from the high risk group R4. The affected sheep was homozygous for the allele PrP^{ARQ} (ARQ/ARQ) coding for alanine (A), arginine (R) and glutamine (Q) at three most relevant codons (136, 154 and 171, respectively. The healthy sister of the positive ewe was heterozygous in the PrP locus and carried alleles ARQ/ARR.

Key words: scrapie; prion; PrP gene; polymorphism; Slovakia

Introduction

Scrapie is a neurodegenerative disese of the TSE group, most commonly affecting adult sheep and goats (Novák *et al.*, 2000). TSE are associated with conformational conversion of a normal host-encoded cellular prion protein PrP^C into a misfolded conformer PrP^{SC}. Accumulation of proteinase-resitant PrP^{SC} in central nervous system (CNS) is a histological and biochemical hallmark of scrapie and other TSE.

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Abbreviations: BSE = bovine spongiform encephalitis; CNS = central nervous system; ELISA = enzyme-linked immunosorbent assay; PK = proteinase K; PrP = prion protein; TSE = transmissible spongiform encephalitis

The histopathological detection of protein aggregates called scrapie-associated fibrils in the brain of sheep represents one of the criteria indicating the infection with scrapie (Merz *et al.*, 1981). However, over the last 10 years tremendous progress has been made in the field of scrapie diagnostics. The recent screening assays for TSE, approved by the European Union, are based on specific immunochemical detection of proteinase-resistant PrP in the brain of affected animals (Moynagh and Schimmel, 1999). Because of the structural and antigenic conservation of PrP protein between mammalian species, rapid screening tests for bovine PrP^{SC} (e.g. the Prionics Check and Enfer TSE) are used also for detection of ovine PrP^{SC}.

In the present study we report a sheep scrapie diagnosed in an ewe of Merino breed from southeastern Slovakia. The presence of protein-resistant PrP conformer in the brain was proved by two different laboratories using two different immunochemical screening tests. In addition, the genotyping

analysis of the PrP gene polymorphism in the positive sheep was performed by nucleotide sequencing.

Materials and Methods

TSE screening for proteinase-resistant PrP in sheep brain tissues was performed by two different immunochemical tests, Western blot analysis using the Prionics Check (Prionics AG) and ELISA using the Enfer TSE (Abbott). Both rapid screening tests were carried out according to the manufacturers' instructions

PCR. To amplify PrP DNA, genomic DNA was extracted with the DNeasy Tissue Kit (Qiagen) from an aliquot of tissue homogenate. A complete coding region for sheep PrP was amplified by PCR in an automated cycler Perkin Elmer 2400 from genomic DNA using suitable primers (sense 5'-ATGGTGAAAAGCCA CATGG-3' and antisense 5'-CCTCATTTTTCTCAAGTAGATAG-3' (Belt *et al.*, 1995), which generated a DNA fragment of 770 bp. Proofreading Vent DNA polymerase (NEB) and the following conditions for PCR reaction were employed: 95^0C/3 mins, followed by 35 cycles of 94^0C/1 min, 56^0C/1 min, and 72^0C/90 secs, and final extension of 72^0/7 mins.

The PCR product was subjected to electrophoresis in 1% agarose gel under standard conditions (90 V, 30 min) and stained with ethidium bromid.

The resulting PCR product of 770 bp was excised from the gel, purified by column chromatography (QIAquick Gel Extraction Kit, Qiagen) and subjected to nucleotide sequencing.

Nucleotide sequencing of the DNA fragment was carried out with ABI Prism™ 377 Perkin Elmer Sequencer and Big Dye Terminator Kit (Applied Biosystems). The primers were the same as those used for PCR. The data of DNA sequencing were evaluated by the Sequence Navigator Program (Perkin Elmer).

Results and Discussion

Scrapie, the first known natural form of TSE, has been reported worldwide as occurring in most sheep producing regions. We diagnosed the first confirmed scrapie in Slovakia during a routine screening for TSE which is obligatory for sheep over 9 months of age assigned for human consumption. The positive sheep was a 60-month-old ewe of Merino breed raised in a farm in a southeastern part of the country. It is known that scrapie occurs most frequently in sheep of either sex between 2 and 5 years of age (Dickinson, 1976), the modal age of clinical onset being about 3.5 years (Parry, 1983; Wineland *et al.*, 1998). However, the affected animal did not exhibit any clinical signs of scrapie. The asymptotic appearance of the positive animal points out the relevance of the compulsory screening for scrapie. It is interesting that reports of a scrapie-like condition in the continental Europe in 17–18th century primarily link scrapie to imported Spanish Merino sheep (Parry, 1983).

The Western blot analysis performed using the Prionics Check in the State Veterinary Laboratory in Zvolen revealed the presence of proteinase K-resistant fragments of PrP protein in the investigated sheep brain tissue (data not shown). This positive finding was confirmed in the National Reference Laboratory for TSE in Bratislava by both Western blot analysis (the Prionics Check) and ELISA (the Enfer TSE). Fig. 1 shows a characteristic three-band pattern of proteinase K-resistant sheep PrP in Western blot analysis. In confirmatory ELISA, the positive sheep brain sample gave 909 LU while the negative control gave only 0.9 LU (values >5.5 LU were considered positive) (Table 1).

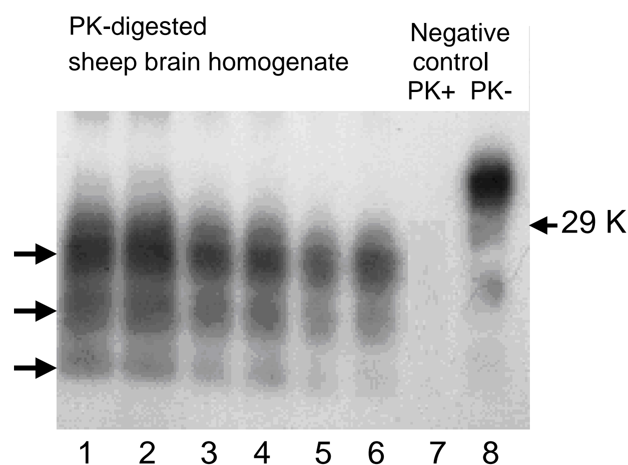

Fig. 1

Demonstration of PK-resistant PrP in the brain of positive sheep by Western blot analysis

Sheep brain homogenate, 10 μl (lanes 1 and 2); sheep brain homogenate, 5 μl (lanes 3 and 4); sheep brain homogenate, 2 μl (lanes 5 and 6); PK-digested negative (healthy) control (lane 7); PK-untreated negative control (lane 8). The arrows on the left side show characteristic three-band pattern of PK-resistant PrP fragments. The arrow on the right side shows position of the 29 K marker.

Table 1. Analysis of the brain tissue from the affected ewe by Enfer TSE ELISA

Sample	Results (LU)		
	Replicate 1	Replicate 2	Average
Sheep brain	975.70	843.17	909.43
Positive control	1869.42	1765.29	1817.35
Negative control	0.80	1.07	0.93

LU = light unit. Values >5.5 LU are considered positive.
Blank values were 1.25–1.30.

Table 2. Genotyping of the PrP locus of positive ewe and its healthy sister and deduced amino acids at polymorphic positions in PrP

Genotyped sheep	Polymorphic positions with alternative amino acids											
	112 M/T	127 G/S	**136 A/T/V**	137 M/T	138 S/A	141 L/F	143 H/R	151 R/C	**154 R/H**	**171 Q/H/R**	176 N/K	211 R/Q
Positive ewe												
Allele 1:	M	G	**A**	M	S	L	H	R	**R**	**Q**	N	R
Allele 2:	M	G	**A**	M	S	L	H	R	**R**	**Q**	N	R
	Genotype ARQ/ARQ (risk group R4)											
Healthy sister												
Allele 1:	M	G	**A**	M	S	L	H	R	**R**	**Q**	N	R
Allele 2:	M	G	**A**	M	S	L	H	R	**R**	**R**	N	
	Genotype ARQ/ARR (risk group R3)											

A = alanine, C = cysteine, F = phenylalanine, G = glycine, H = histidine, K =lysine, L =leucine, M = methionine, N = asparagine, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine.

In sheep, the PrP gene polymorphism resulting in variability of 15 amino acids at 12 positions (112,127, 136, 137, 138, 141, 143, 151, 154, 171, 176, and 211) has been observed (Tranulis *et al*., 1999). A correlation in genetic susceptibility of sheep to scrapie with the PrP gene polymorphism has been recognized. In particular, the polymorphism identified at codons 136, 154 and 171 play a crucial role regarding natural scrapie in sheep (Hunter *et al*., 1996). According to the PrP polymorphism at positions 136, 154 and 171, sheep are classified into five risk groups, from R1 to R5 (Distl, 2000). Homozygous carriers of the PrP allele $A^{136}R^{154}R^{171}$ (A and R stand for alanine and arginine, respectively) represent the risk group R1, most resistant to scrapie.

In contrast, the animals homozygous for the allele $V^{136}R^{154}Q^{171}$ (V and Q stand for valine and glutamine, respectively) are most susceptible to scrapie and belong to the highest risk group R5. Therefore the genotyping analysis of the PrP gene derived from the tissue of positive sheep was performed. The DNA fragment containing the complete coding sequence of the sheep PrP gene was PCR-derived and directly sequenced. Then the corresponding primary structure of the protein was deduced and the amino acids at polymorphic positions were determined (Table 2). According to the amino acids at three most relevant positions 136, 154 and 171 the affected ewe was homozygous for the allele $A^{136}R^{154}Q^{171}$ with PrP genotype ARQ/ARQ. The carriers of this genotype are classified into the high risk group R4 and are expected to have a high risk of development of scrapie after the contact with exogenous source of scrapie agent. The genotype ARQ/ARQ has been detected in Slovakia in 20% of sheep in autochthonous Improved Valachian breed (Tkáčiková *et al*., 2003). We performed also analysis of one sister of the positive ewe from the same flock. Immunochemical screenings for TSE by Western blot analysis and ELISA did not reveal the presence of proteinase K-resistant PrP in the brain tissue (data not shown). Genotyping analysis of the PrP locus revealed that the sister was heterozygous for codon 171 coding for glutamine and arginine, respectively, and carried the alleles ARQ/ARR. According to this genotype the ewe could be classified into the risk group R3 with low susceptibility to scrapie. It is interesting that the reported scrapie geographically coincides with one of thirteen bovine spongiform encephalitis (BSE) cases reported until now from Slovakia (data not shown).

In Slovakia, clinically or laboratory proved data of sheep scrapie have not been documented yet. Mitrová *et al*. (1991) have reported histopathological finding of scrapie-associated fibrils in sheep of Valachian breed from geographically distant region of Orava. However, this finding has not been confirmed by the current European Union-approved methods. Therefore the recent report represents the first confirmed sheep scrapie in Slovakia.

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