Slov Vet Res 2004; 41 (2): 83-88
UDC 619:616.831:575.1:636.3

Original Research Paper

SCRAPIE-SUSCEPTIBILITY-LINKED POLYMORPHISMS OF THE PRION PROTEIN GENE IN ISTRIAN PRAMENKA SHEEP

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Summary: Scrapie is a transmissible degenerative disease of the central nervous system occurring naturally in sheep. It belongs to a group of prion diseases known as transmissible spongiform encephalopathies (TSE) and it is characterized by the accumulation of a proteinase-resistant prion protein mainly in the central nervous system. Three main scrapie-linked polymorphisms in the prion protein gene (*Prnp*) that modulate susceptibility to scrapie have been described and are located at the codon positions 136, 154 and 171. In order to evaluate and characterize the *Prnp* polymorphisms in Slovenian autochthonous sheep breeds and to evaluate the genetic susceptibility of these sheep to scrapie, we analyzed the *Prnp* of 41 sheep of the Istrian Pramenka breed from Slovenia. The polymorphisms at codons 136, 154 and 171 were determined by nucleotide sequencing of the *Prnp*. Four allelic variants and eight different genotypes were determined. At codon 136, two codon variants were observed, encoding amino acids alanine (A) and valine (V), 82.9 % of the sheep examined had AA, 2.5 % had VV and 14.6 % had AV. At codon 154, 90.2 % had arginine/arginine (RR) and 9.8 % arginine/histidine (RH); at the codon position 171, 46.3 % had amino acids glutamine/glutamine (QQ), 36.6 % had codon variant QR, and 17.1 % had RR codons. The most frequent genotype in the Slovenian population of Istrian Pramenka sheep is ARQ/ARQ (29.3 %). Animals carrying this genotype are moderately susceptible to scrapie, as shown by studies on other sheep breeds that were naturally or experimentally infected. The allelic variant VRQ, known to carry a very high risk of scrapie is only poorly represented in the population of the examined sheep (9.8 %). The more abundant allelic variant was ARR, which is typical to sheep resistant to scrapie (32.9 %).

Key words: Istrian Pramenka; sheep; scrapie; prion protein gene; polymorphism; susceptibility; sequencing

Introduction

Scrapie is a transmissible neurodegenerative disease of sheep and goats, showing characteristic brain pathology with vacuolated neurons, that generally manifests clinically with symptoms like pruritus, motor disorders and body deterioration. It belongs to a group of disorders known as transmissible spongiform encephalopathies (TSE) or prion diseases. Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS) fatal familial insomnia (FFI), fatal sporadic insomnia (FSI) and variant Creutzfeldt-Jakob disease (vCJD) in humans and bovine spongiform encephalopathy (BSE) in cattle also belong to this group. Common to all TSE diseases is the accumulation of an abnormal prion protein (PrP^{Sc}) in the tissue of the central nervous system. The normal prion protein (PrP^{c}) is expressed in most tissues of the body, with the highest expression in the nervous tissues. Prions seem to be composed exclusively of a modified isoform of PrP designated PrP^{Sc}. The PrP^{c} is converted into PrP^{Sc} through a process whereby a portion of its α-helical and coil structure is refolded into β-sheet. This structural transition causes changes in its physicochemical properties that result in the accumulation of PrP^{Sc} in the cells. In its abnormal isoform, the PrP^{Sc} is infectious and proteinase-resistant (1, 2).

The occurrence of natural scrapie in sheep is a complex interplay between genetic susceptibility and different strains of the infectious prion (1,

Received: August, 2004
Accepted for publication: October, 2004

2, 3). Scrapie-related polymorphisms in the coding region of the prion protein (PrP) gene, *Prnp*, have been studied in number of sheep breeds in various countries (4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14). While a range of amino-acid polymorphisms of the sheep *Prnp* have been described, not all of them have been found to be associated with disease susceptibility. However, the polymorphisms at codon 136 and 171 have been confirmed to be associated with scrapie susceptibility in experimental (10) and natural scrapie; valine at codon 136 and glutamine at codon 171 are often present in the genomes of sheep that are highly susceptible to scrapie (9, 11). For the polymorphism at codon 154, the evidence suggests that histidine at codon 154 is associated with a low susceptibility to scrapie in some breeds of sheep (12).

The Istrian Pramenka is a domestic, autochthonous sheep that was traditionally bred in the regions of Istria and the Karst (in Croatia and Slovenia). It originates from primitive domestic sheep known as "Zackel" sheep (13). This milk sheep is very tenacious, its long strong legs, distinct long-stepping walk and its slim snout enables its adaptation and survival in the rough conditions on the rocky terrain in the Karst region. In 2002, the estimated number of breeding females of Istrian Pramenka sheep in Slovenia was 770 animals, but this number is increasing (http://www.tiho-hannover.de/einricht/zucht/eaap/descript/1617.htm).

The *Prnp* genotypes of Istrian Pramenka sheep breed have not been examined previously. Therefore, we have studied the *Prnp* polymorphisms of the Istrian Pramenka that are known to be associated with scrapie susceptibility.

Material and methods

Animals

A total of 41 Istrian Pramenka sheep, from a larger flock of about 400 adult sheep located in Slovenia's Karst region were examined in the study. No clinical signs of scrapie were ever observed in the animals of the examined flock. The absence of the proteinase-resistant prion protein (PrP^{Sc}) in the obex tissue of the animals that either died or were slaughtered was confirmed by immunochemical screening using either the rapid post-mortem test Prionics Check Western (Prionics AG) (western blot analysis) or an ELISA test Enfer TSE Kit (Abbott).

DNA isolation and Prnp amplification

Genomic DNA was isolated from blood leukocytes or from frozen brain tissue by a standard phenol-chloroform protocol as described by Sambrook et al. (18) or with a Wizard Genomic DNA Purification Kit (Promega).

The *Prnp*, including the whole open reading frame (794 bp), was amplified by a polymerase chain reaction (PCR) with set of primers: SPrP-1 (5'-CATCATGGTGAAAAGCCACATAGGC-3') and SPrP-2 (5'-GAAAACAGGAAGGTTGCCCCTATCC-3') as described by Ikeda et al. (6). The conditions for the PCR amplification were: initial step at 94 °C for 2 min; denaturation at 94 °C for 1 min; annealing at 58 °C for 1 min; and extension at 72 °C for 2 min. The PCR products were electrophoresed in a 0.8 % agarose gel containing ethidium bromide (all Sigma) and visualised under ultraviolet light.

Sequencing

The PCR products were purified from the agarose gel using Wizard® SV Gel and a PCR Clean-Up System (Promega). The purified PCR products were directly sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Kit with the primers: SPrP5: 5'-ATAAGCTGGGATTCTCTCT-3' SPrP-2: 5'-GAAAACAGGAAGGTTGCCCTATCC-3' as described by Gombojav (14). The sequencing was performed on an ABI Prism 310 autosequencer.

Data analyses

The DNA sequence data were analysed using Chromas (Technelysium).

Results

The western blot analyses and ELISA tests performed on the tissues of the animals that died or were slaughtered were always negative for the proteinase-resistant prion protein (PrP^{Sc}).

We observed dimorphisms at codons 136, 154 and 171; the different codon frequencies are shown in Table 1. At codon 136 – A and V were observed, at codon 154 – R and H were observed and at codon 171 – Q and R were determined. Four different allelic variants were observed along

Table 1: Codon frequencies for *Prnp* in Istrian Pramenka sheep

Codon position	136			154			171		
Codon variant	AA	AV	VV	RR	RH	HH	QQ	QR	RR
Number	34	6	1	37	4	0	19	15	7
Percentage	82.9	14.6	2.5	90.2	9.8	0	46.3	36.6	17.1

Table 2: Allelic frequencies for *Prnp* in Istrian Pramenka sheep

Allele (136/154/171)	Number of animals	Percentage
ARQ	43	52.4
ARR	27	32.9
VRQ	8	9.8
AHQ	4	4 9

Table 3: Genotypes of *Prnp* in Istrian Pramenka sheep associated with scrapie susceptibility

Scrapie susceptibility	Number of animals	Percentage
Low (ARR/ARR; ARQ/ARR; AHQ/ARR)	20	48.8
Moderate (ARQ/ARQ; ARQ/AHQ)	14	34.1
High (VRQ/VRQ; ARQ/VRQ)	5	12.2
Occasional occurrence (ARR/VRQ)	2	4.9

with eight different PrP genotypes. Tables 2 and 3 show the allelic and genotypic frequencies of the examined animals. The histidine observed at position 154 was never in the homozygous form.

The frequencies of the genotypes associated with susceptibility to scrapie are shown Figure 1. The most frequent genotype was ARQ/ARQ (29.3 %), which is moderately susceptible to scrapie. Only 12.1 % of Istrian Pramenka population were shown to be highly susceptible to scrapie (ARQ/VRQ and VRQ/VRQ) (Table 3).

Discussion

The results of our study show the frequencies of different codons for the amino acids at positions 136, 154 and 171 of the PrP protein in the autochthonous Istrian Pramenka sheep breed,

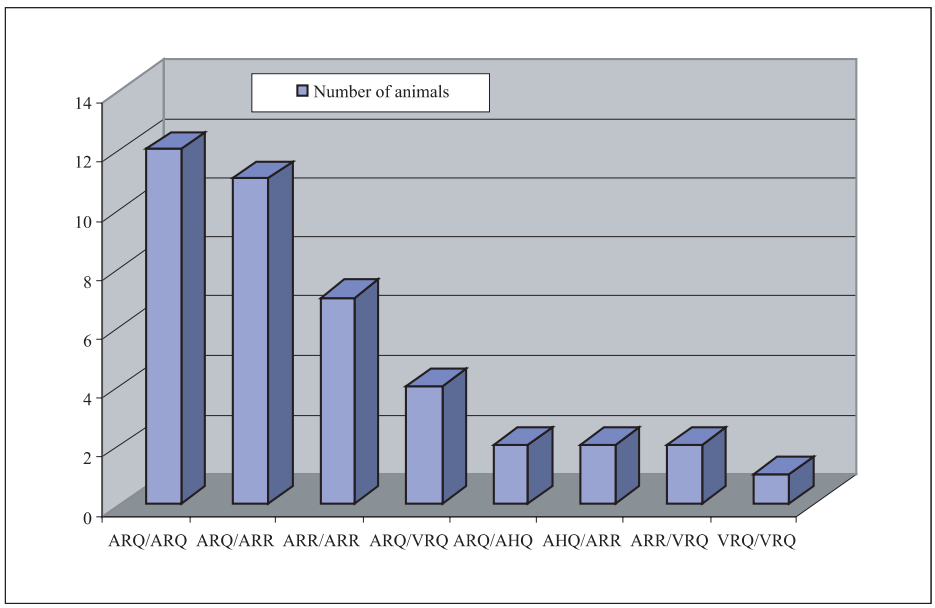

Figure 1: Graphic presentation of Prnp genotype frequencies in the examined Istrian Pramenka sheep

their allelic variants and genotypes. At codon 136 – A and V were observed, at codon 154 – R and H were observed and at codon 171 – Q and R were determined. As has also been the case for other sheep breeds, not all the theoretically possible allelic and genotypic variants were observed. Despite this, the Istrian Pramenka sheep belongs, in terms of *Prnp* genetics, to a group of breeds that are genetically complex having four allelic variants and eight different genotypes, similar to Cheviot, Swaledale and Shetland sheep that also have the same four *Prnp* alleles (19). The most frequent combination of the three codons in the Slovenian population of the Istrian Pramenka was ARQ (52.4 % allelic frequency; Table 2) and the most frequent genotype was ARQ/ARQ (29.3 %; Figure 1). The animals with this genotype are moderately susceptible to scrapie, as was shown by studies on other sheep breeds that were naturally or experimentally infected (9, 10, 11). Fortunately, the allelic variant VRQ, which is known to carry a very high risk of scrapie, was poorly represented in the population of the examined sheep (9.8 %). However, this ratio was still above a recently reported average of 3.7 % from a population of 430233 sheep from 243 different breeds (20). In the examined group of sheep, the homozygous form of the allele was only found in one animal (2.4 %). The allelic variant ARR, which is typical to sheep resistant to scrapie, was better represented in the examined sheep population (32.9 %).

The genetic data on the *Prnp* polymorphisms offers the possibility of controlling natural scrapie infections in the sheep population by selecting genotypes carrying scrapie-resistant alleles in breeding animals. Once the genotype is determined it may be possible to breed sheep resistant to scrapie by eliminating the *Prnp* genes with codon forms 136-V and 171-Q, which have been shown to influence scrapie-susceptibility (21). Breeding programmes for animals carrying genotypes that determine resistance and the elimination of animals with VRQ alleles are already underway in many European countries. The VRQ-allelic variant was poorly represented in the examined sheep breed, therefore its elimination could be possible. However, considering the high frequency of the ARQ-allelic variant in the Istrian Pramenka sheep population, eliminating all the susceptible sheep could be difficult and should be viewed as a long-term process. Additionally, atypical forms of scrapie have also been reported in sheep having genotypes that are known to be resistant to classical scrapie (22). Therefore, consideration should be given to the use of an appropriate breeding programme coupled with the use of diagnostic tests to control the possible onset of scrapie in our population of Istrian Pramenka sheep.

Scrapie in Istrian Pramenka sheep has never been diagnosed in Slovenia. The results of our study show that the majority of the Istrian Pramenka sheep population in Slovenia has the genotype which is moderately susceptible, however

some of them also carry the highly susceptible VRQ alleles. Considering the genotypes determined, we could expect the onset of scrapie in the Istrian Pramenka sheep population in Slovenia, therefore special attention should be paid to the monitoring for possible clinical signs of scrapie and to screen any suspect animals for the presence of PrP^{Sc}.

Acknowledgements

We thank Mrs. Magdalena Dobravec for her technical assistance. This research was supported by the Slovenian Ministry of Agriculture, Forestry and Food and the Slovenian Ministry of Education, Science and Sport (project number CRP V4-0758-02).

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POLIMORFIZMI GENA ZA PRIONSKI PROTEIN, ODGOVORNI ZA DOVZETNOST ZA PRASKAVICO PRI ISTRSKIH PRAMENKAH

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Povzetek: Praskavica je prenosljiva degenerativna bolezen centralnega živčnega sistema ovac, znana tudi kot oblika prenosljive spongiformne encefalopatije (transmissible spongiform encephalopathy - TSE), s katero se ovce okužijo po naravni poti. Spada v skupino prionskih bolezni, za katere je značilno, da se v celicah kopiči na proteazno delovanje odpornei prion, kar se pojavlja predvsem v centralnem živčnem sistemu. Poznani so trije najpomembnejši polimorfizmi gena za prionski protein (*Prnp*), ki modulirajo dovzetnost ovac na praskavico; nahajajo se na kodonih 136, 154 in 171. V programu določanja polimorfizmov gena za PrP in ovrednotenja genetske dovzetnosti slovenskih avtohtonih pasem ovac za praskavico, smo analizirali *Prnp* pri 41 ovcah pasme istrska pramenka. Polimorfizme na kodonu 136, 154 in 171 smo določali s sekvenciranjem nukleotidnih zaporedij gena *Prnp.* Ugotovili smo štiri alelne različice in osem genotipov. Na kodonu 136 smo ugotovili dve različici aminokislin, alanin (A) in valin (V). Pri 82,9 % pregledanih živali smo ugotovili alanin/alanin (AA), 2,5 % pregledanih živali je imelo različico VV, 14,6 % pa AV. Na kodonu 154 smo pri 90,2 % ugotovili arginin/arginin (RR), pri 9,8 % živali pa arginin/histidin (RH). Na položaju 171 smo pri 46,3 % preiskovanih živali določili aminokislino glutamin/glutamin (QQ), 36,6 % je imelo različico QR, 17,1 % pa RR. Pri slovenski populaciji istrskih pramenk je najpogosteje zastopan genotip ARQ/ARQ (29,3 %), ki je značilen za ovce, srednje dovzetne za praskavico, kar so pokazale preiskave naravno ali poskusno okuženih ovac. Alelna različica VRQ, ki je značilna za ovce z visokim tveganjem obolevanja za praskavico, je le slabo zastopana v populaciji preiskanih ovac (9,8 %). Bolje je zastopana alelna različica ARR, ki je značilna za živali, odporne na praskavico (32,9 %).

Ključne besede: istrska pramenka; ovca; praskavica; gen za prionski protein; polimorfizem; dovzetnost; sekvenciranje