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THE IDENTIFICATION OF DNA POLYMORPHISM AT THE 154 CODON OF THE OVINE PRNP GENE BY ALLELIC DISCRIMINATION USING A DUAL FLUORESCENT PROBE ASSAY

IDENTIFIKÁCIA DNA POLYMORFIZMU V KODÓNE 154 PRNP GÉNU OVIEC METÓDOU ALELICKEJ DISKRIMINÁCIE POUŽITÍM DUÁLNEJ FLUORESCENČNEJ PRÓBY

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The aim of this study was to investigate prion gene polymorphism at codon 154 of common breeds of sheep in Slovakia to provide information about their susceptibility to scrapie. The material involved 82 sheep. Genomic DNA was extracted from whole blood by salting out method. Genetic analysis of the PrP gene was realised by an allelic discrimination technique using the TaqMan assays. In sheep population the predominace of homozygote genotype RR was detected. Heterozygote genotype RH was present in breed Lacaune (1 animal) and crossbreeds Improved Valachian × Lacaune (2 animals) and Improved Valachian × Lacaune × East Friesian Sheep Breed (1 animal). Homozygote genotype HH was not observed.

Keywords: sheep, allelic discrimination, scrapie, PRNP, codon 154

Transmissible spongiform encephalopathies (TSE) or prion diseases represent a group of transmissible neurodegenerative diseases that includes Creutzfeldt-Jakob disease (CJD), variant CJD of man, bovine spongiform encephalopathy (BSE) of cattle, and scrapie of sheep and goats. The key event in TSE is the post-translational modification of a host-encoded cellular protein, named the prion protein (PrP), into a pathological isoform (PrP^{Sc}) that accumulates in the brain of affected subjects (Prusiner, 1982; Vaccari et al., 2009).

Scrapie is a disorder characterized by the deposition of the prion protein (PrP^{Sc}; associated with TSE) in the central nervous system and lymphoreticular system. Characteristic clinical signs of the disease are behavioural disturbances, pruritus and increased difficulty in locomotion. Studies of naturally and experimentally scrapie-affected sheep have shown that genetic susceptibility to the disease is modulated by allelic variation in the PrP gene (Hunter, 2000, Acín et al., 2004). In sheep, it is known that polymorphisms at codons 136 (A/V), 154 (R/H) and 171 (R/H/Q) of PRNP, the gene encoding PrP, are associated with TSE resistance/susceptibility (Poucke et al., 2005). Their combination gives five commonly detected alleles: ARR, ARH, ARQ, AHQ, VRQ [11]. The effect of the different alleles on scrapie susceptibility is complex: both the allelic frequency and the influence of each allelic variant vary among ovine breeds. Sheep carrying the VRQ/VRQ genotype are considered the most susceptible to the disease, whereas sheep of ARR/ARR genotype seem to be resistant (Vascellari et al., 2005). The association between the polymorphism at codon 154 (arginine R or histidine H) and susceptibility to scrapie is still unclear since some data suggest that a histidine at codon 154 offers some protection in certain breeds of sheep (L´Homme et al., 2007).

The diagnostic methods currently applied to detect a TSE infection (BSE rapid tests as well as confirmatory methods) are based on the detection of PrP^{Sc}. The four commonly used rapid tests as well as the confirmatory methods [SAF immunoblot and immunohistochemistry (IHC)] that have been recommended by the Office International des Epizooties (OIE) apply polyclonal or monoclonal antibodies to detect PK-treated PrP^{Sc} accumulated in the brains of TSE-affected animals (Buschmann et al., 2004). Comparable haematologic status of sheep are not useful in diagnosis of scrapie (Williams, 2005; Rolinec et al., 2012). It is generally accepted that the susceptibility of sheep to scrapie is directly linked to particular allelic polymorphisms of PrP (Buschmann et al., 2004). Genotyping sheep for genes that appear to confer resistance to contracting classical scrapie has been used for several years in an attempt to eradicate scrapie through selective breeding. Currently, there are numerous methods for detecting single nucleotide polymorphisms (SNP) in genes. Many of these use real-time PCR methodologies for detecting the SNP (Johnson et al., 2007).

The aim of this study was to investigate prion gene polymorphism at codon 154 of interest in common breeds of sheep in Slovakia to provide information about their susceptibility to scrapie.

Material and methods

The material involved 82 sheep of two breeds (Lacaune and Tsigai) and four crossbreeds (Tsigai × Lacaune, Improved Valachian × Lacaune, Improved Valachian × Lacaune × East Friesian Sheep Breed and Lacaune × East Friesian Sheep Breed). Ovine genomic DNA was extracted from blood samples by salting out method (Miller et al., 1987). Genetic analysis of the PrP gene was realised by an allelic discrimination technique using hydrolysis probe TaqMan. The assay was set up for the analysis of codon 154 to discriminate between R (arginine – susceptibility to scrapie) and H (histidine – partially resistant to scrapie). Allelic discrimination technique consists in the amplification of specific regions of DNA using couples of primers not marked and fluorochromes-labelled TaqMan probes specific for the mutations relative to the interested loci. The sequences of primers and probes described by Vascellari et al. (2005) are shown in Table 1. The reaction

Table 1 Primers and probes used for the analysis of PrP polymorphism of codon 154 by the allelic discrimination assay

Name (1)	Position (2)	Sequence (5´ to 3´) (3)
Primers (4)		
154-For	(503-523)	TGGCAATGACTATGAGGACCG
154-Rev	(575-552)	TGGTCTGTAGTACACTTGGTTGGG
TaqMan MGB probes (5)		
154 – Arg (FAM)	(526-541)	ACTATCgTGAAAACAT
154 – His (VIC)	(525-542)	TACTATCaTGAAAACATG

Tabuľka 1 Primery a próby použité pre analýzu PrP polymorfizmu kodónu 154 použitím metódy alelickej diskriminácie
(1) názov, (2) pozícia, (3) sekvencia (5´-3´), (4) primery, (5) TaqMan MGB próby

mixture in total volume 25 µl contained 2 × Maxima Probe qPCR Master Mix without ROX (Fermentas), 0.3 µM of each primer, 0.2 µM of each probe and 10 ng DNA template. The PCR amplification and allelic discrimination assay were carried out using the real-time thermocycler Rotor-Gene 6000® (Qiagen) following standard two step thermal profile by Vascellari et al. (2005). The fluorescence is measured at each amplification cycle by using Rotor-Gene 6000 Series Software 1.7. Using two probes (one for each nucleotide) marked with two different fluorochromes, it is possible to discriminate at the same time the presence of the 2 alleles. The specificity of the probes allows only the perfect alignments between probe and filament to produce a fluorescent signal. In the case of mismatch (a different base of the DNA mould and sample) the hybridisation is strongly inhibited with a consequent negligible release of fluorescence. The result of this reading is the classification in 2 categories: homozygote (samples with one of the 2 alleles) and heterozygote (samples with both the alleles).

Resuls and discussion

The PrP gene variability at codon 154 in sheep was carried out by allelic discrimination assay. This technique uses two probes; the first, specific for the allele R is labelled with FAM and the second, specific for the allele H, is labelled with VIC. In either assay, a substantial increase in FAM fluorescence indicates a homozygous RR, a substantial increase in VIC fluorescence indicates a homozygous HH, and intermediate increase in both signals usually indicates a heterozygote RH. The emitted fluorescence was collected during the step of annealing the PCR cycle. At the end of the amplification, a Rotor-Gene 6000 Series Software 1.7 identified the marker components and established the contribution of each one, allowing the identification of the genotypes. The patterns of results from the real-time allelic discrimination assay for the homozygous genotype RR and heterozygous genotype RH for codon 154 are shown in Figure 1. To help score the genotypes the Rotor-Gene software allows endpoint fluorescence values for the two dyes to be automatically corrected for background and plotted against each other in bi-directional scatter plots (Figure 2). The clustering of samples in scatter plots in addition to the real-time fluorescence traces gives easy and accurate genotype scoring.

The predominace of homozygote genotype RR was detected in sheep population. Heterozygote genotype RH was present in breed Lacaune (1 animal) and crossbreeds Improved Valachian × Lacaune (2 animals) and Improved Valachian × Lacaune × East Friesian Sheep Breed (1 animal). Homozygote genotype HH was not observed. Detailed genotype and gene frequencies per breed and crossbreed are presented in Table 2.

The allele R has been reported due to its high association with delayed onset of symptoms for classical scrapie strains. The effect of allele H on scrapie remains controversial, because

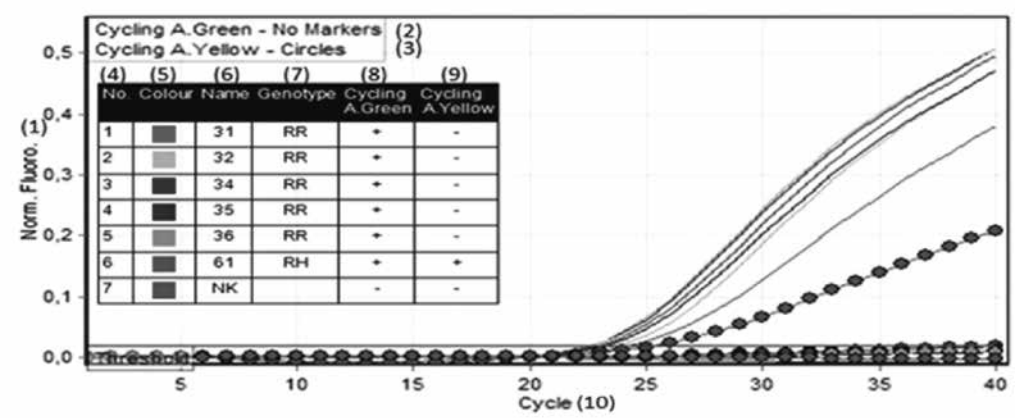

Figure 1 The patterns of results from the real-time allelic discrimination assay for the homozygous genotype RR and heterozygous genotype RH for codon 154
——— FAM labelled probe (R allele); —o—o—o— VIC labelled probe (H allele)

Obrázok 1 Schéma výsledkov z real-time analýzy alelickej diskriminácie pre homozygotný genotyp RR a heterozygotný genotyp HR kodónu 154
(1) normalizovaná fluorescencia, (2) cyklovanie zeleným kanálom – bez značky, (3) cyklovanie žltým kanálom – s krúžkom, (4) číslo, (5) farba, (6) názov, (7) genotyp, (8) cyklovanie zeleným kanálom, (9) cyklovanie žltým kanálom, (10) cyklus
——— FAM značená próba (R alela); —o—o—o— VIC značená próba (H alela)

Figure 2 Representative results of scatter plot analysis of TaqMan assay
Obrázok 2 Reprezentatívne výsledky rozptylu TaqMan analýzou
(1) cyklovanie zeleným kanálom, (2) cyklovanie žltým kanálom, (3) homozygot RR, (4) heterozygot RH, (5) bez templátu, (6) homozygot HH

Table 2 Frequency of genotypes and alleles of 154 codon in the population of sheep breeds

Breed (1)	Total (2)	Genotype frequencies (3)			Allelle frequencies (4)	
		RR	RH	HH	R	H
LC	36	0.9722	0.0278	0	0.9681	0.0139
T	15	1	0	0	1	0
T/LC	14	1	0	0	1	0
IV/LC	13	0.8462	0.1538	0	0.9231	0.0769
IV/LC/VF	3	0.6667	0.3333	0	0.8333	0.1667
LC/VF	1	1	0	0	1	0
TOTAL(5)	82	0.9512	0.0488	0	0.9756	0.0244

Breed: LC – Lacaune, T – Tsigai, IV – Improved Valachian, VF - East Friesian Sheep Breed
Plemeno: LC – lacon, C – cigaja, ZV – zošľachtená valaška, VF – východofrízska ovca

Tabuľka 2 Genotypové a alelové frekvencie kodónu 154 v populácii ovčích plemien
(1) plemeno, (2) celkom, (3) genotypové frekvencie, (4) alelové frekvencie, (5) celkom

it presented protection against scrapie in some breeds (Hunter, 1997), but it was susceptible in other breeds (Acutis et al., 2006) and even presented a positive risk to Nor98 scrapie (Colussi et al, 2008, Guan et al., 2011). The allele R was absolutely predominant (97.56%) in all breed sheep and only four heterozygotes with H were detected. RR and RH genotypes across all breeds were 0.9512 and 0.0488 respectively. These data were consistent with observations in other studies. In Spain, Acín et al. (2004), found the R allele the most common when analysing sheep from different breeds. The frequencies of allele R were predominated in the breeds of Rasa Aragonesa and Ansotana (97.3%, 97.8 %, respectively). In population of the Churra Tensina breed there was slight superiority of allele R (66.7 %). In the breeds of Ojinegra, Cartera, Maellana, Roya Biebilitana only allele R was found. The high superiority of the allele R (99.44 %) in population of Chines Hu sheep was reported by Guan et al. (2011). Analysing 358 sheep from 3 breed, Harrington et al. (2009) also found the RR genotype to be the most common. The second most observed genotype in all breeds was RH. The HH genotype was present only in one animal in Rambouillet breed.

Conclusion

It may be concluded that in the population of all sheep breeds codon 154 of PrP gene with a superiority of genotype RR and a superiority of allele R is present. It seems that Slovak sheep have a low risk to scrapie according to this allele distribution, which seems to delay the progression of scrapie. Genetic structure examined in population of all sheep breeds remained within the range quoted in literature for other cattle breeds.

Súhrn

Cieľom štúdie bolo preskúmať polymorfizmus prion proteínového génu v kodóne 154 plemien oviec bežne chovaných na Slovensku s cieľom poskytnúť informácie o ich vnímavosti ku scrapie. Materiál zahŕňal 82 oviec. Genomická DNA bola izolovaná zo vzoriek krvi vysolovacou metódou. Genetická analýza PrP génu bola realizovaná metódou alelickej diskriminácie použitím TaqMan próby. V populácii bola detegovaná prevaha homozygotného genotypu RR. Heterozygotný genotyp RH bol prítomný pri plemene lacaune (1 zviera) a pri krížencoch

zošľachtená valaška × lacaune (2 zvieratá) a zošľachtená valaška × lacaune × východofrízska ovca (1 zviera). Homozygotný genotyp HH nebol pozorovaný.

Kľúčové slová: ovce, alelická diskriminácia, scrapie, PRNP, kodón 154

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