Slovak J. Anim. Sci., 43, 2010 (1): 40 - 44

ISSN 1337-9984

Short communication

DETECTION OF OVINE PRION PROTEIN POLYMORPHISM IN CODONS 136, 154 AND 171 BY PCR-PRIMER INTRODUCED RESTRICTION ANALYSIS

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ABSTRACT

Amino acid polymorphism in the prion protein gene at codons 136, 154 and 171 affect the susceptibility of sheep to scrapie, a naturally occurring form of transmissible spongioform encephalopathy (TSE). The aim of this work was the development of a reliable and cost-effective method for ovine prion protein polymorphism in codons 136, 154, and 171. Blood and hair roots from breeding rams of Improved Valachian, Tsigai and Lacaune breeds were tested as a source of DNA for PCR. The established polymerase chain reaction-primer introduced restriction analysis (PCR-PIRA) enables identification of all important genotypes used for marker-assisted breeding of sheep for decreased classical scrapie susceptibility. For validation of this method 91 animal samples with known genotypes were analysed by PCR-PIRA with total agreement. Reported method does not require high purity of DNA and tissue lysate can directly be used for PCR. No expensive equipment is required.

Key words: genotyping; ovine; PCR; scrapie

INTRODUCTION

The resistance or susceptibility of sheep to scrapie, a naturally occurring form of transmissible spongioform encephalopathy (TSE) seems to be influenced by polymorphism in the prion protein (PrP) gene (*PRNP*) linked to the variations at codons 136, 154 and 171 (Hunter, 1997). Polymorhism at these codons is the basis for the marker-assisted breeding of sheep for decreased scrapie susceptibility in many countries. Various PCR-based approaches have been used to determine polymorphism at codons 136, 154 and 171 including direct sequencing (Tranulis et al., 1999), RFLP (Hunter et al., 1993; Elsen et al., 1999; Yuzbasiyan-Gurkan et al., 1999; Lühken et al., 2004), DGGE (Belt et al., 1995), allele-specific oligonucleotide hybridization (Ishiguro et al., 1998), primer extension assay (Vaccari et al., 2004), SSCP (Zhou et al., 2005) and Real-Time qPCR (Simek et al., 2007).

Sheep breeding programme for scrapie resistance in Slovakia routinely use the PCR-SSCP method for sheep genotyping. The PCR-SSCP technique scans the whole amplified sequence and unkown allelic variation can complicate interpretation of the results and subsequently require sequencing. Polymerase chain reaction–primer introduced restriction analysis (PCR–PIRA) is a method

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Received: December 15, 2009
Accepted: March 24, 2010

that can be used for detecting a single nucleotide mutation in any gene without a restriction site around the mutation site (Haliassos, et al., 1989; Jacobson and Moskovits 1991). The PCR-PIRA is easy to perform and the nucleotide sequence at specific positions are detected.

The aim of this work was the development of an alternative cost-effective screening method for ovine prion protein gene polymorphism and verify the reliability of the test. Two PCR-PIRA analyses were developed to determine all PRNP haplotypes occuring due to the variations at codons 136, 154 and 171. The sequenced DNA samples with known nucleotide sequences at selected codons (136, 154, 171) were used for development of this PCR-PIRA method.

MATERIAL AND METHODS

PrP genotype for all samples used in this work was previously determined at the State Veterinary and Food Administration by PCR-SSCP method. Blood and hair roots were tested as a source of DNA for sheep genotyping. Blood samples (38) were obtained from 15 breeding rams of Improved Valachian breed, 20 rams of Tsigai and 3 rams of Lacaune breed. Samples of hair roots (53) were selected from previously genotyped breeding rams for FecB (23 from Improved Valachian and 30 from Tsigai breed).

The samples were analyzed for the amino acids encoded at positions 136 (A/V), 154 (R/H) and 171 (Q/R/H) of the PrP using restriction fragment length polymorphism (RFLP).

Two hundred microlitres of whole blood samples were first washed twice in 1 ml of red blood cells lysis buffer (final concentration 155 mM NH_4Cl; 10 mM $KHCO_3$ and 0.1 mM EDTA). Cell pellet was resuspended in 100 µl of 1x PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4) without $MgCl_2$, suplemented with 1% Triton X-100 and 400 ng/µl proteinase K. Five to 10 hair roots were overlaid with 50 µl of the same buffer as a blood cell pellet. The samples were incubated at 55°C for 30 minutes, then at 96°C for 5 minutes and cooled to room temperature. Samples were briefly vortexed and centrifuged at 16060 x g for 1 minute. The touch-down PCR conditions (PTC-200 DNA Engine; MJ Research) were 94°C for 2 min, 4 cycles of 94°C for 20 s, 64°C for 30 s (-0.5°C per cycle), 72°C for 30 s, 31 cycles of 94°C for 20 s, 62°C for 30 s, 72°C for 30 s with a final extension at 72°C for 5 min, in 1x PCR buffer (20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0 at 25°C) with final a concentration of 1.5 mM $MgCl_2$ 0.4 µM of forward and reverse primers, 250 µM dNTPs, 1U Platinum Taq DNA Polymerase (Invitrogen) in total volume of 50 µl.

Two different PCR fragments of 530 bp were amplified using the same forward primer PRP_F: 5‘-CAC ATA GGC AGT TGG ATC CTG GTT CTC-3‘, which is nucleotide 22290 to 22316 of GenBank sequence U67922 and with a modified reverse primer PRP_QR: 5‘-TCA TGC ACA AAG TTG TTC TGG TT**c ag**A TA-3‘ corresponding to nucleotides 22819 to 22791 of GenBank sequence U67922 and creating an artificial restriction site for *Alw*N I when the codon CAG for glutamine occurs at position 171. The other modified reverse primer PRP_RR: 5‘-TCA TGC ACA AAG TTG TTC TGG TTA CTA TA**t**-3‘ corresponding to nucleotides 22819 to 22790 of GenBank sequence U67922 and creating an artificial restriction site for *Bsp*D I in the case of the codon CGA for arginine at position 171.The mismatching bases are lower case bold typed.

PCR product (10 µl) was digested with appropriate restriction enzyme (New England Biolabs, 5 U per sample; Table 1) at least 4 hours at 37°C or overnight. PCR fragment amplified with PRP_F and PRP_QR primers was digested with restriction enzyme *Alw*N I for analyzing codon 171 for glutamine. When the codon CAG occurs at position 171 digestion resulted for 340 bp, 164 bp and 26 bp fragments. PCR product obtained with PRP_F and PRP_RR primers was double digested with restriction enzymes *Bsp*D I and *Bsp*H I simultaneously. In both fragments, the codon for valine at position 136 and for histidine at position 154 form restriction sites for *Bsp*H I. Double digestion of PRP_F and PRP_RR primers amplified fragment resulted in several fragments. For valine at position 136, 395 bp and 135 bp fragments and for histidine at position 154, 447 bp and 83 bp fragments were created with *Bsp*H I restriction enzyme. In the case of the codon CGA for arginine at position 171, 498 bp and 32 bp fragments were created with *Bsp*D I restriction enzyme (see Table 1).

The digested DNA was electrophoretically separated on 3.5% agarose gels containing ethidium bromide at 15V/cm in 10mM lithium borate buffer, pH 8.0. The products were visualized under UV light and photographed using a GDS 8000 (UVP) camera.

RESULTS AND DISCUSSION

The PCR resulted in amplification of 530 bp long fragments of ovine prion gene covering all analysed codons. Identification of different alleles was done by restriction digestion and subsequent agarose gel electrophoresis (Figure 1a and 1b).

In both fragments the codon for valine at position 136 and for histidine at position 154 form restriction sites for *Bsp*H I. Pattern of restriction fragments digestion of the PCR product obtained with primers PRP_F and PRP_QR with *Alw*N I and double digestion of the PCR product obtained with primers PRP_F and PRP_RR with *Bsp*H I and *Bsp*D I simultaneously are presented in Table 1.

Figure 1a

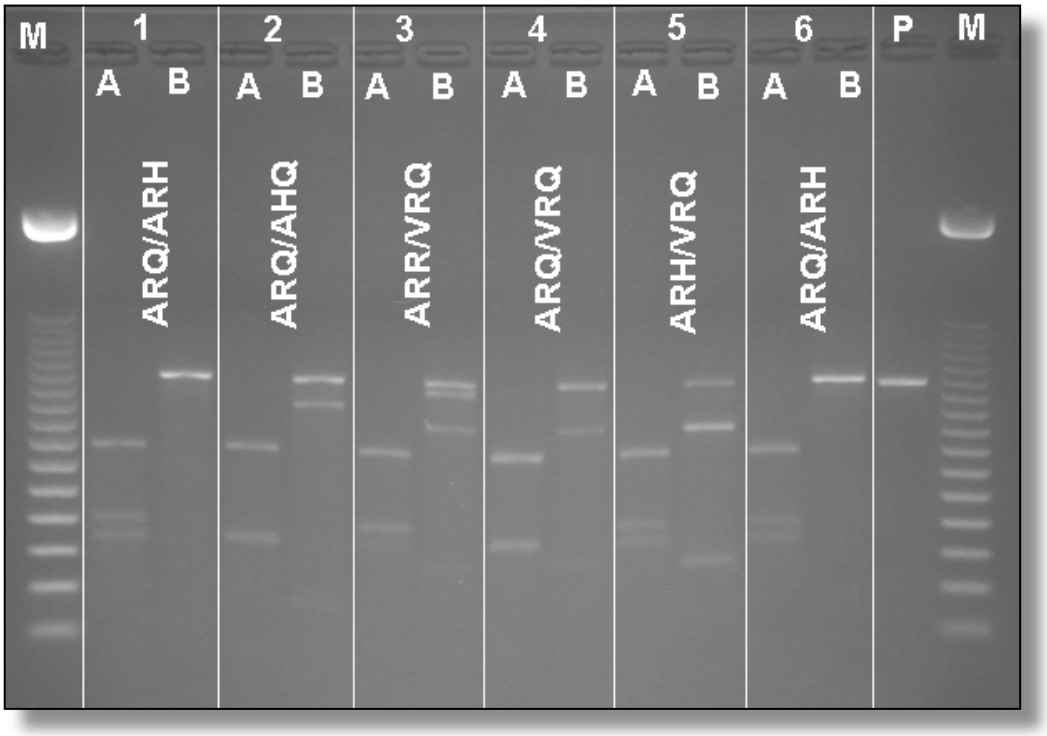

Figure 1b

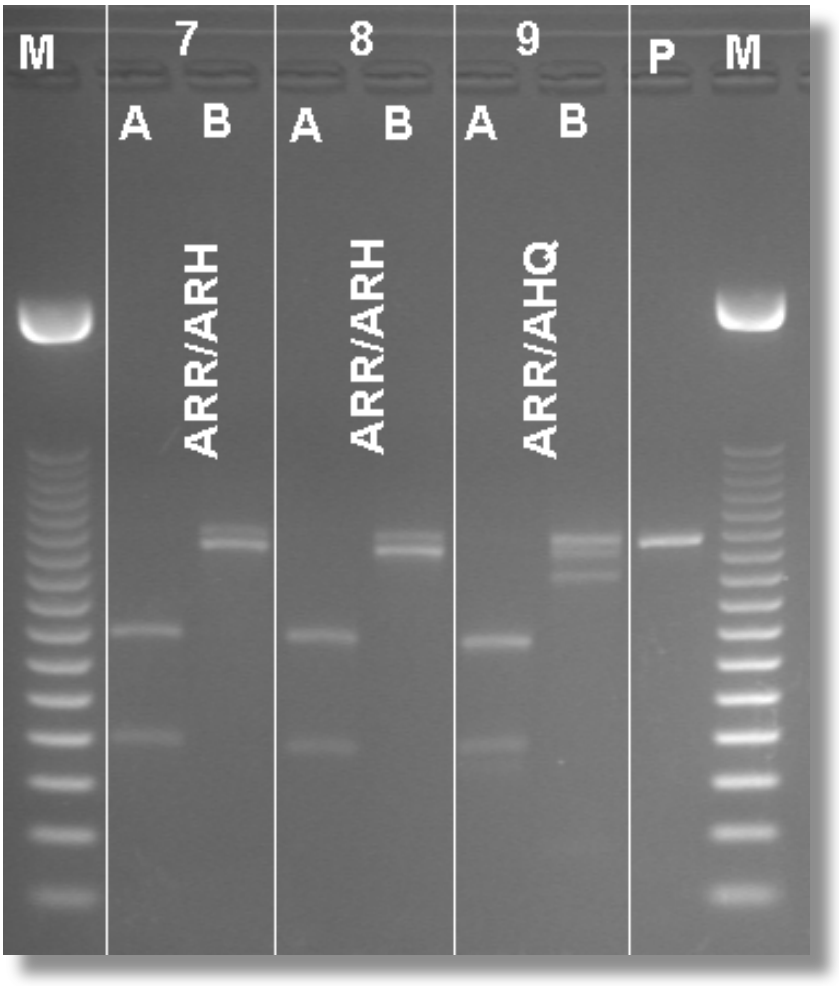

Figure 1a, 1b. Ovine PrP genotyping by PCR-PIRA. Samples from sheep with different genotypes (1-9) separated by agarose electrophoresis and stained with ethidium bromide. Lane A: PCR product (PRP_F+PRP_ QR) digested with *Alw*N I, Lane B: PCR product (PRP_F+PRP_RR) digested with *Bsp*D I and *Bsp*H I, M: molecular weight marker: 50 bp DNA Step Ladder (Promega, G4521), P: non-digested PCR product (530 bp). The length of fragments are given in Table 1. The genotype is determined by identification of restriction fragments in lane A and B for each sample

Table 1: PCR primers combination, pattern of the restriction fragments and haplotype of the ovine prion protein gene in selected codons

PCR primers	Restrition enzyme	Restriction fragments (bp)	Haplotype: 136 154 171
PRP_F + PRP_QR	*Alw*N I *Bsp*D I *Bsp*H I	340, 190 530 530	A R R A R H
	*Alw*N I *Bsp*D I *Bsp*H I	340, 164, 26* 530 530	A R Q
	*Alw*N I *Bsp*D I *Bsp*H I	340, 164, 26* 530 447, 83	A H Q
	*Alw*N I *Bsp*D I *Bsp*H I	340, 164, 26* 530 395, 135	V R Q
PRP_F + PRP_RR	*Alw*N I *Bsp*D I *Bsp*H I	340, 190 498, 32* 530	A R R
	*Alw*N I *Bsp*D I *Bsp*H I	340, 190 530 530	A R H A R Q
	*Alw*N I *Bsp*D I *Bsp*H I	340, 190 530 447, 83	A H Q
	*Alw*N I *Bsp*D I *Bsp*H I	340, 190 530 395, 135	V R Q

* Note: small fragments of 26 bp, 32 bp are not seen on the gel

The advantage of the established PCR-PIRA method is that it enables distinction between all important genotypes used for marker-assisted breeding of sheep for decreased scrapie susceptibility. Other nucleotide polymorphisms may occur within the coding region of *PRNP* but they are not included in eradication programmes yet. Using PRP_F and PRP_QR primers this method allows direct detection of 171 CAG codon (Q), and is not influenced by 3‘ end of the primer sequence in the amplified PCR product in this case. Both amplified fragments contain an internal control restriction site for *Alw*N I restriction enzyme, for enzyme activity.

Lühken et al. (2004) described PCR-RFLP method to determine all PRNP haplotypes occuring due to variations at codons 136, 154 and 171. In this method after digestion 196 bp PCR product of samples with ARR/VRQ and ARR/AHQ genotypes, an additional 196 bp fragment occurs, but without diagnostic relevance. This PCR-RFLP method has been incorporated in breeding programmes concerning Merinoland sheep breed.

Twelve sequenced DNAs were used for development of this PCR-PIRA method for screening polymorphism of the ovine prion protein gene. An important criterion for use of this method in routine genetic testing is reliability of the test. Thus the PrP genotype of 91 breeding rams was determined by PCR-PIRA and compared with results obtained previously, as per the established protocol of the State Veterinary and Food Administration. The results of both methods were in total agreement.

Hair or tissue lysate can be directly used for PCR. No expensive equipment for sequencing or real-time qPCR or fluorescent labeled probes are required. No polyacrylamide gel preparation, manipulation and optimalization of the SSCP conditions are needed.

In conclusion, described method is suitable for rapid and cost-effective screening of the ovine prion protein gene polymorphism in breeding programmes for eradication of such haplotypes sensitive for classical form of scrapie. Both PCR products can be used for screening of L/F polymorphism in codon 141 by *Mnl* I restriction enzyme, which is in some breeds associated with atypical scrapie (Moum et al., 2005).

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Agriculture of the Slovak Republic (research task 2006 UO 27 091 05 02 091 05 13).

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