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Application of temperature-gradient gel electrophoresis for detection of prion protein gene polymorphisms in Polish Świniarka sheep

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Abstract. This study presents preliminary data on the polymorphism in the prion protein gene of Świniarka sheep using temperature gradient gel electrophoresis (TGGE). Available data indicate that sensitivity to scrapie is associated with polymorphisms in three codons of prion protein gene: 136,154, and 171. The TGGE method was used to detect point mutations in these codons responsible for sensitivity or resistance to scrapie. This study revealed presence of an allele encoding valine (V) in codon 136, which is associated with high sensitivity to scrapie and occurred in the form of heterozygous allele together with alanine (AV). The highest variability was observed in codon 171, with presence of arginine (R) and glutamine (Q) in the homozygous (RR or QQ) as well as the heterozygous form (RQ). The results of examination of fifty sheep DNA samples with mutations in codons 136, 154, and 171 demonstrated that TGGE can be used as a simple and rapid method to detect mutations in the PrP gene of sheep. Several samples can be run at the same time, making TGGE ideal for the screening of large numbers of samples.

Key words: Gene polymorphism; sheep; temperature-gradient gel electrophoresis.

Scrapie is a fatal neurodegenerative disease of small ruminants belonging to the group of transmissible spongiform encephalopathies (TSEs). The TSEs are characterized by the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) in the central nervous system and in lymphoid tissues of affected individuals.[1,2,4,6,10] The gene encoding PrP is located on chromosome 13q 17–18 and consists of three exons. However, only exon 3 encompasses the entire open reading frame of the PrP gene. Its translation produces the protein consisting of 256 amino acids. The remaining exons contain nontranslating sequences (Fig. 1).

Early epidemiological observations of sheep scrapie indicate that sensitivity or resistance to scrapie is associated with variability at codons 136, 154, and 171 of the PrP gene. Of the five most frequent scrapie alleles (ARR, AHQ, ARH, ARQ, and VRQ), ARR is thought to be associated with resistance and VRQ with susceptibility to the disease.[3,5,6] Susceptibility to scrapie is mainly associated with presence of valine (V) at codon 136 and glutamine (Q) at codon 171; homozygotes (VV_{136} and QQ_{171}) are

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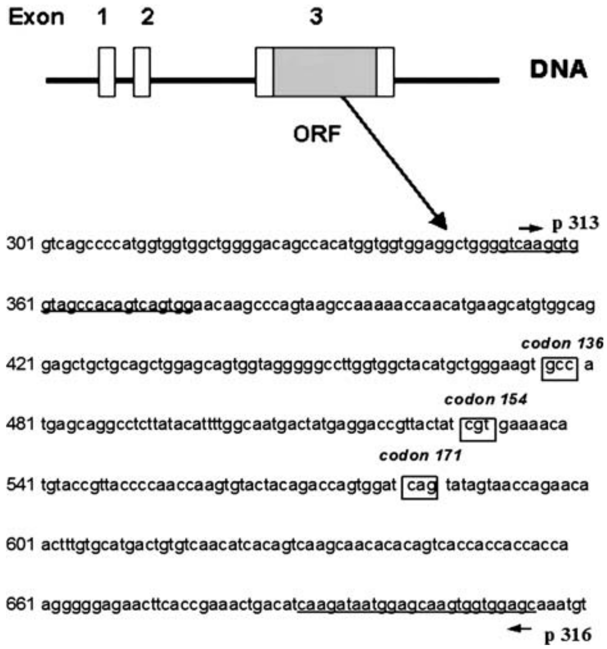

Figure 1. Schematic diagram of the genomic organization of the PrP gene and the fragment of nucleotide sequence of sheep exon 3 of PrP gene with oligonucleotides used to amplify the 445 bp fragment. Underlined nucleotide sequences indicate the positions of PCR primers (p313 and p316). Open boxes represent the codons responsible for variability of sheep PrP gene (codons 136, 154, 171).

particularly susceptible. On the other hand, presence of alanine (A) at codon 136 and arginine (R) at codon 171 is associated with high resistance to scrapie.[4,11] Based on this knowledge, the United Kingdom launched a National Scrapie Plan in July 2001 aiming at reducing and eventually eliminating scrapie from the national sheep flock. Several other countries, including France, Italy, and the USA, started implementing a similar breeding program.[5,10]

The size of the Polish sheep national flock is estimated at 360,000. According to European Union (EU) legislation, even a small national flock like Poland's requires genotyping of a representative number of animals each year. For this purpose, the test method needs to combine high sensitivity in detecting sequence variations in the three target codons with ability to simultaneously screen a large number of samples. Combination of polymerase chain reaction (PCR) and temperature gradient gel electrophoresis (TGGE) fulfills these requirements. The TGGE method exploits two physical properties of DNA: the effect of nucleic acid sequence on temperature-induced unwinding of DNA strands and the influence of partial denaturation on electrophoretic mobility.[16] Each point mutation changes the melting temperature of the PCR product and its electrophoretic mobility. Accordingly, DNA molecules with single base pair differences undergo partial denaturation at temperatures clearly lower than the temperature required for strand separation and migrate slower in gel.[7,9,13,16] Thus, TGGE enables separation of DNA fragments of the same size but with different melting temperatures resulting from a single base pair difference. The method can also be applied for routine analysis of many samples in a fairly short time. TGGE is a modification of denaturing gradient gel electrophoresis, where the chemical denaturing agents (formamide and urea) are replaced by a thermal one.[9,16] This paper describes the TGGE method as a screening technique for studying sequence polymorphisms of sheep PrP gene.

As a preliminary study aimed at providing data on genotypes of Polish sheep, one pedigree sheep flock belonging to a primitive Świniarka breed in southern Poland was selected as a representative flock of native sheep. The reason for selection of this unique breed is that it appears to be of ancient local origin, and so far Poland is considered a scrapie-free country. Therefore, native sheep breeds would be expected to harbor scrapie-resistant genotypes.

Peripheral blood samples from 50 sheep were collected in tubes with 5 mM EDTA as anticoagulant. Genomic DNA was extracted from samples using a commercial kit.[a] The PCR primers used in this study were essentially as previously described,[11] but were modified by the addition of a 40-bp GC clamp at the 5′ end of each oligonucleotide (Table 1). The reaction mixture was 25 µl in volume and was composed of 2.5 µl of 10× PCR buffer (100 µM Tris-HCl, pH 8.8, 500 µM KCl),[b] 1.5 mM $MgCl_2$, 50 µM deoxynucleoside triphosphates, 0.5 µM of each primer, and 0.5 unit of *Taq* polymerase.[b] DNA (0.5 µg) was added to the reaction mixture, and amplification was performed as follows: denaturation at 94°C for 4 min followed by 30 cycles of 30-sec denaturation at 94°C, 30-sec primer hybridization at 66°C, 30-sec elongation at 72°C, and a final 7-min elongation. Amplification products (445-bp fragment) were analyzed on 2% agarose gels containing ethidium bromide (0.4 µg/ml) and visualized under ultraviolet radiation. The correct product size was compared with a size ladder (100-bp ladder).[c]

Polymorphisms at codons 136, 154, and 171 of the PrP gene were identified by TGGE,[d] which was performed in two steps. First, electrophoresis was conducted at a constant temperature of 20°C and 250V for 7 min; second, a temperature gradient from 40°C to 60°C was applied parallel to the migration direction and electrophoresis was

Table 1. PCR primers used in TGGE analysis.

Primer	Sequence
313†	5′-(GC 40)*GTCAAGGTGGTAGCCACAGTCAGTGG-3′
316†	5′-(GC 40)*GCTCCACCACTCGCTCCATTATCTTG-3′

* (GC 40)GC-clampCGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG. † Nucleotide sequences of primers were described by O'Doherty et al.[11]

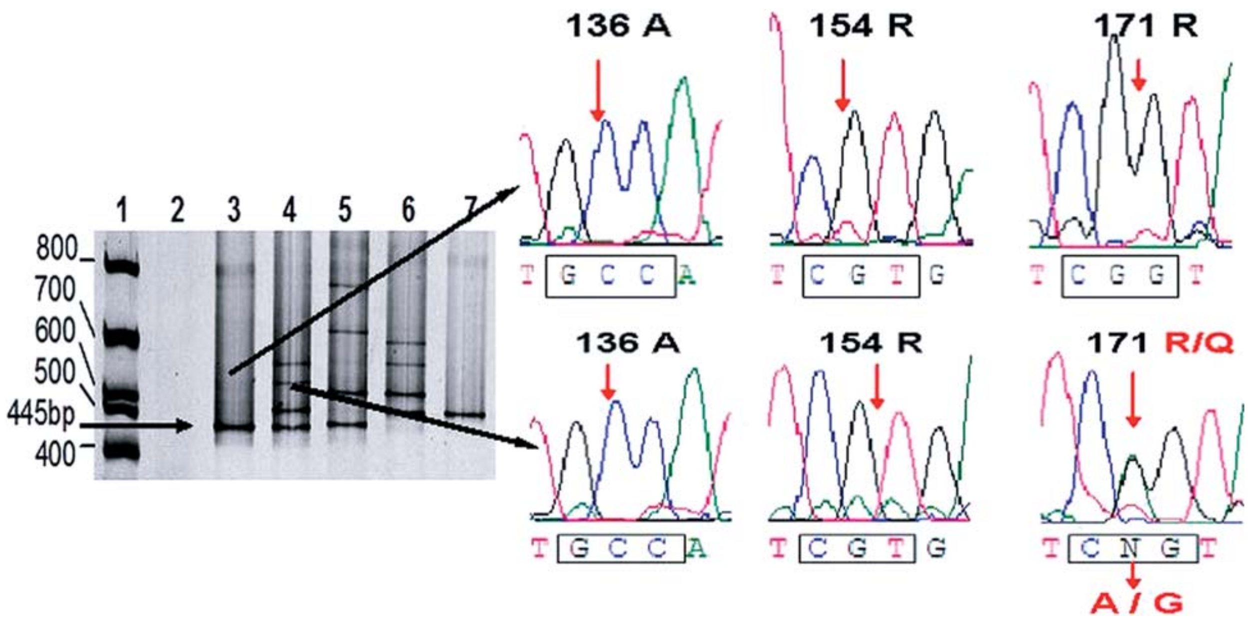

Figure 2. Electrophoresis of PCR products of PrP gene of control samples from Czarnogłówka breed and fluorograms of sequencing of codons 136, 154, 171 (genotype ARR/ARR and ARR/ARQ). Temperature gradient (40°C→60°C) is same as the direction of the electric field. Lane 3 (genotype ARR/ARR), no mutation in both alleles yields a single band with the highest melting temperature. Transition A→G at codon 171 in one allele (genotype ARR/ARQ; lane 4) changes the melting temperature of the DNA and yields four bands (2 heteroduplex and 2 homoduplex). Lane 1, 100-bp ladder; lane 2, negative control (no DNA added); lane 3, homozygote ARR/ARR (wild type 445bp); lane 4, heterozygote ARR/ARQ; lane 5, heterozygote ARR/AHQ; lane 6, heterozygote ARQ/AHQ; lane 7, homozygote ARQ/ARQ.

continued at 250V for 40 min. After the electrophoresis, the gels were silver-stained using 0.3% silver nitrate to visualize the bands. Electrophoresis of the amplification products was performed on an 8% polyacrylamide gel (acrylamide/N,N-ethylenebisacrylamide 37.5 : 1)[e] containing 8 M urea and 2% glycerol. Electrophoresis buffer was 1× TAE pH 8.3.[f] About 3 ng of DNA was loaded into each slot of the 8% polyacrylamide gel. Gel electrophoresis mobility and stability of PCR amplified PrP gene fragments were calculated using online software (http://www.biophys.uniduesseldorf.de/local/POLAND/poland.html). As controls, DNA samples from Czarnogłówka sheep with known genotypes were used. These five different genotypes (ARR/ARR, ARR/ARQ, ARR/AHQ, ARQ/AHQ, and ARQ/ARQ) confirmed by sequencing served as the reference genotypes (Fig. 2).

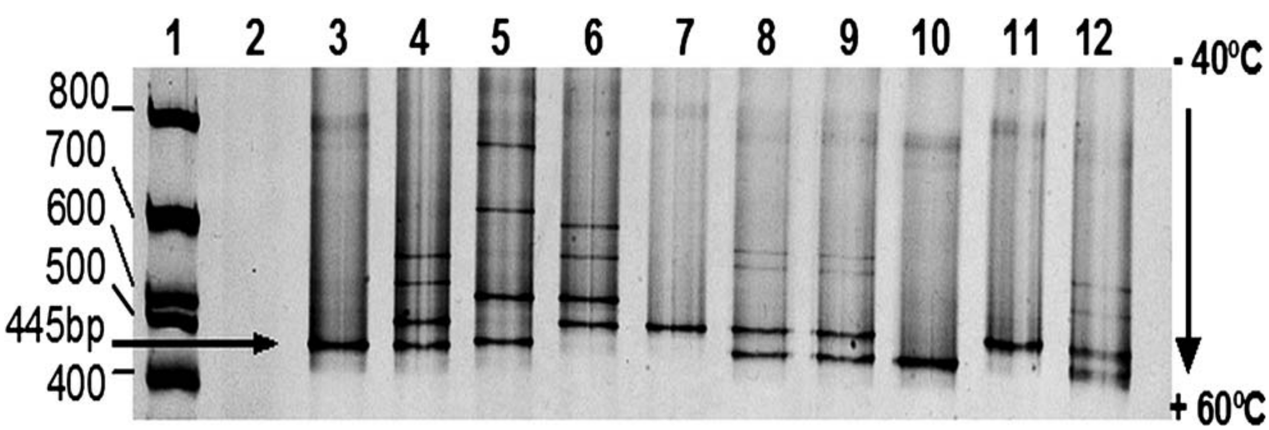

Figure 3. Electrophoresis of PCR products of sheep PrP gene. Temperature gradient (40°C→60°C) is same as the direction of the electric field. Lane 1, 100-bp ladder; lane 2, negative control (no DNA added); lanes 3, 4, 5, 6, 7, positive controls of five different genotypes from Czarnogłówka breed of sheep (line 3, ARR/ARR genotype; lane 4, ARR/ARQ genotype; lane 5, ARR/AHQ genotype; lane 6, ARQ/AHQ genotype; line 7, ARQ/ARQ genotype). Lanes 8, 9, 10, 11, 12, samples from Świniarka breed of sheep (lane 10, ARR/ARR genotype; lane 11, ARQ/ARQ genotype; line 12, ARR/ARQ genotype). Band patterns (lanes 8 and 9) different from control samples reflects existence ARR/VRQ genotype (confirmed by sequencing). The slightly different migration distance between the hetero- and homozygous samples reflects differences in nucleotide sequence.

Table 2. Mutations in PrP codons 136,154,171 and genotypes of Świniarka sheep.

Mutations (amino acids)			Genotype	Sheep number (%)
codon 136	codon 154	codon 171		
—	—	—	$AA_{136}RR_{154}RR_{171}$	22 (44)
—	—	CGG→CAG (Arg→Gln)	$AA_{136}RR_{154}RQ_{171}$	15 (30)
GCC→GTC (Ala→Val)	—	CGG→CAG (Arg→Gln)	$AV_{136}RR_{154}RQ_{171}$	9 (18)
—	CGT→CAT (Arg→His)	CGG→CAG (Arg→Gln)	$AA_{136}RH_{154}RQ_{171}$	2 (4)
—	—	CGG→CAG (Arg→Gln)*	$AA_{136}RR_{154}QQ_{171}$	2 (4)

* Transition G→A was found in both alleles as compared to the wild sequence of PrP gene.

To evaluate the reliability of TGGE, analysis of several randomly selected samples of Świniarka sheep was performed at the DNA Sequencing and Oligonucleotide Synthesis Facility of the Institute of Biochemistry and Biophysics in Warsaw, Poland. Furthermore, sequencing of DNA samples with different TGGE band patterns than 5 control samples (Fig. 3; lanes 8, 9) was performed to verify their genotype. In every case, the results of PrP genotyping obtained with the TGGE method were identical to those obtained by sequencing.

DNA fragments with the same sequence are easily discernible because they migrate during electrophoresis over the same distance from the starting point. A single band in Fig. 3 (lane 11; homozygote ARQ/ARQ) reflects the existence of a point mutation in both alleles of the same codon. A G→A transition at codon 171 decreases the melting temperature and gel mobility of the DNA fragment in comparison with the wild-type fragment (lanes 3, 10; homozygote ARR/ARR). The presence of four bands in one lane reflects the existence of a point mutation on one allele, which creates additional heteroduplex bands visible above the homoduplex bands (Fig. 3). The slower migrating bands are hybrids of wild-type and mutant fragments containing mismatched base pairs. The distance between the heteroduplex bands as well as between heteroduplex and homoduplex bands depends on position and number of point mutations (Fig. 3; Table 2).

Of the 50 sheep genotyped, 41 were homozygous AA_{136} and the others were heterozygous for AV_{136} (Table 2). Variation at codon 154 (RH_{154}) was found only in two cases. The remaining 48 sheep were homozygous for RR_{154}. The greatest genetic variation was observed at codon 171, and two of the three possible alleles were detected: of the 50 sheep, 22 were homozygous for RR_{171} (44%), two were homozygous for QQ_{171} (4%), and 26 were heterozygous for RQ_{171} (52%). No H alleles were found at codon 171.

This is the first study of the frequency of PrP gene polymorphisms in the Polish population of Świniarka sheep, a very primitive breed of sheep. This study confirmed that using the TGGE method it is possible to detect more than one point mutation within the PrP gene.[10,14,17] Band patterns of DNA are characteristic for given genotypes of PrP gene and depend on the number and location of particular mutations. The TGGE method is a reproducible and sensitive technique and can be useful in routine screening of sheep flocks. In comparison with the widely used single-strand conformation polymorphism (SSCP) analysis, the interpretation of TGGE gels is facilitated by the appearance of heteroduplex bands. In contrast to TGGE, the interpretation of SSCP gels is sometimes difficult due to poor resolution of the bands, which depends greatly on the length of the fragments and the use of different running conditions.[9]

The results of this study indicate that the dominant alleles in Świniarka sheep are ARR and ARQ. These PrP alleles are also frequently present in Romanov sheep,[4] Suffolk sheep[8,11] and Valachian sheep.[14] The ARR allele is associated with high resistance to scrapie, so high frequency of this allele in some breeds of sheep can be exploited to increase the genetic resistance to scrapie of sheep flocks through introduction of selective breeding programs. Scrapie has not yet been reported in Poland, and the introduction of a scrapie-resistance breeding program could diminish the risk of its outbreak. Analysis of sheep belonging to the Świniarka breed also revealed the presence of the VRQ allele, which is associated with high susceptibility to scrapie. This allele is very rare or absent in some sheep breeds, like Berichone du Cher,[5] Masses,[10] and Suffolk,[8,11] but highly frequent in Icelandic sheep (about 80%) and in Norwegian Rygja sheep (48%).[2] There is no data on PrP genotypes of Świniarka sheep in currently available literature, probably because this breed of sheep can be found only in the southern and southeastern part of Europe.

In conclusion, the occurrence of the VRQ allele in Świniarka sheep indicates that this breed cannot be considered as scrapie-resistant. A more extensive screening program is needed to provide more comprehensive data. Świniarka sheep, being well adapted to local environmental conditions, can constitute the basis for future breeding programs as a donor of scrapie-resistant genotype.

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Sources and manufacturers

a. DNA Isolation Kit for Mammalian Blood, Roche Diagnostics, GmbH, Mannheim, Germany.
b. Fermentas AB, Vilnius, Lithuania.
c. GeneRuler™ 100bp DNA Ladder Plus, Fermentas AB, Vilnius, Lithuania.
d. Biometra, GmbH, Göttingen, Germany.
e. Acrylamid-Bis ready-to-use solution 40%, Merck KGaA, Darmstadt, Germany.
f. TAE buffer 10x pH 8.3 Tris-acetace-EDTA buffer, Merck KGaA, Darmstadt, Germany.

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