APPLICATION OF TEMPERATURE GRADIENT GEL ELECTROPHORESIS METHOD FOR STUDYING THE SHEEP PRION PROTEIN (PrP) GENE POLYMORPHISM

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This study presents the preliminary data on the polymorphism of the PrP gene using two breeds of sheep occurring in Poland, i.e. Wrzosówka and Świniarka. Available data indicate that presence of allele encoding valine at codon 136 in many breeds of sheep is associated with high sensitivity to scrapie. Our study did not reveal presence of this allele in the examined sheep. The highest variability was observed in codon 171:RR\textsuperscript{171} associated with resistance to scrapie and in codon QQ\textsuperscript{171}, correlated with sensitivity to the disease.

Key words: sheep, scrapie, prion protein, polymorphism, TGGE.

Scrapie is a fatal neurodegenerative disease of sheep and goats belonging to the group of transmissible spongiform encephalopathies (TSEs) or prion diseases (1, 5-10, 13-14). This group of disorders include also bovine spongiform encephalopathy (BSE) in cattle, infectious encephalopathy of mink (TME), chronic wasting disease of elk and mule-deer in the USA as well as Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in humans (1, 2). There is also theoretical possibility that in countries where BSE was registered the disease might affect some sheep through contaminated feed and up to now there is no method available which might differentiate between natural scrapie and feed borne BSE. Clinical symptoms can be also similar.

Scrapie was first recognized on Shetland Islands more than 200 years ago. Increased incidence of scrapie in early 80-s resulted in introduction in 1984 of control and elimination program by Shetland Flock Health Association (SFHA) (1, 13). Scrapie is an O.I.E. notifiable disease since 1993 (10, 12, 13). Like in other spongiform encephalopathies the main pathological event responsible for the development of clinical symptoms of the disease is the deposition of the pathological, protease resistant form (PrP\textsuperscript{sc}) of the host-encoded protein (PrP\textsuperscript{c}) in the brain (1, 2, 6, 10, 11, 14). This is associated with the loss of neuronal cells leading to the characteristic spongiform changes. Pathological form of PrP accumulates also in the lymphatic tissue (11, 13).
PrP encoding gene is located on chromosome 13q 17-18 and consists of 3 exons. However, only exon 3 encompasses the whole open reading frame of PrP gene. Its translation produces the protein consisting of 256 amino acids. The remaining exons contain non-translating sequences.

Among all amino acid polymorphisms described for codons 112, 136, 137, 138, 141, 151, 154, 171 and 211 only those associated with codons 136, 154 and 171 are considered as having modulating effect on the resistance or susceptibility to the scrapie (6, 10, 11, 14). Among this codons 5 different scrapie related alleles were found i.e.: ARR, AHQ, ARH, ARQ and VRQ, where ARQ allele is probably of wild type (3, 5, 9, 10) (Table 1). The remaining mutated alleles originate probably from wild type allele.

Sensitivity to scrapie associated with variability in codon 136 was evidenced both under natural and experimental conditions and presence of alanine or valine rendered sheep susceptible to the disease. Polymorphism in codon 171 and particularly homozygosity QQ was associated with experimental scrapie in Cheviot sheep and natural scrapie in Suffolk sheep. Codon 154 was found to be less important in modulating sensitivity to scrapie and presence of histidine or arginine was associated with small degree of sensitivity to scrapie in some sheep breeds (6, 7, 10, 11, 13, 15). Taken together the presence of ARR and AHQ alleles was correlated with small sensitivity to scrapie contrary to high risk scrapie associated ARQ and VRQ alleles.

Poland is considered up to date as scrapie free country based mainly on lack of any reports about clinical symptoms of scrapie. The program of registration of all breeded animals is just in the process of setting up and it is obvious that not all dead sheep are examined by veterinary officers. Also the current regulation of the Minister of Agriculture require monitoring of only 7% of slaughtered sheep. Accordingly there is no obvious evidence that scrapie indeed does not exist in Poland. What is more scrapie has been registered in neighbouring countries (8, 16), what makes higher the probability of finding the disease in Poland.

Up to date no evidence of PrP genotyping of sheep breeded in Poland was found and therefore this study has been undertaken. The present article describes only preliminary data in this subject using Wrzosówka and Świniarka breeds as model system and can be starting point for planning more complex screening of PrP genotype among sheep breeds in Poland.

Material and Methods

Peripheral blood from the jugular vein of randomly selected 20 Wrzosówka and Świniarka ewes was collected by venipuncture in to tubes with 5 mM EDTA as anticoagulant. Blood samples were used as a source of DNA for subsequent polymerase chain reaction (PCR) amplification.

PCR. DNA for PCR was isolated using the DNA Isolation Kit for Mammalian Blood, (Roche, Mannheim, Germany). Concentration and purity of DNA preparation was estimated spectrophotometrically. In addition samples of DNA in which the 260/280 absorbance ratio below 1.5 was evidenced were additionally purified using phenol-chloroform-isoamyl alcohol and subsequent ethanol precipitation method. Oligonucleotide primers for PCR were chosen according to O’Doherty et al. (11) and subsequently modified by addition a 40 mer GC clamps at the 5’ end of each oligonucleotide (Table 2). The composition of reaction mixture at a volume of 25 µl
was: 1 x polymerase buffer, 1.5 mM MgCl₂, 0.5 µM of each primer, 50 µM dNTP, and 0.5 unit of Taq polymerase. All the reagents were from Fermentas AB, Vilnius, Lithuania. DNA (0.5 µg) was added to the reaction mixture and amplification process was performed in a programmed thermal cycler (UnoII, Biometra Ltd, Germany). The reaction was started with denaturation at 94°C for 4 min followed by 30 cycles of 30 s denaturation at 94°C, 30 s primer hybridization at 66°C and 30 s elongation at 72°C. The amplification was finished with a 5 min elongation. Amplification products were detected electrophoretically in a 2% agarose gel.

**Genotype analysis.** Genotyping of PrP gene has been performed using method of the temperature gradient gel electrophoresis (TGGE) on Biometra apparatus, Göttingen, Germany. Before perpendicular electrophoresis was started, equal volumes of PCR products from 2 known genotypes of Wrzosówka breed (sheep No. 3135 and sheep No. 117) were mixed in a screwcapped tube and treated in a denaturation/renaturation cycle in order to transfer the base pair exchange into mismatches. The tubes were incubated at 95°C for 5 min and then cooled gradually to 50°C for 15 min. PCR products were then run on an 8% polyacrylamide gel (acrylamide/N,N'-ethylenebisacrylamide 37.5:1) including 8 M urea and 2% glycerol. Cross-linked PCR products in the amount of 50 ng were loaded into a slot at the top of the gel, before the gel was submerged in 1 x TAE pH 8.3 electrophoresis buffer.

Gel electrophoretic mobility and stability of PCR amplified PrP gene fragments were calculated with the program POLAND.

For the first 7 min the electrophoresis was performed at the constant temperature 20°C and 250 V. After this initial step the temperature gradient, from 35°C to 70°C was applied and the electrophoresis has been continued at 250 V for 45 min. Finally the gels were stained using a Silver Staining Kit (Kucharczyk, Techniki Elektroforetyczne, Nadarzyn, Poland) to visualise the bands.

Basing on the optimal temperature range in which the separation of DNA molecules was observed the best temperature gradient for parallel electrophoresis was established and about 3 ng of DNA was loaded in to each slot of the 8% polyacrylamide gel. The initial step was performed in the same way as in the case of perpendicular electrophoresis and then the process was continued in 40-60°C temperature gradient at 250 V for 40 min. Then silver staining was performed.

**Sequencing.** All DNA samples used for genotyping with TGGE method were sequenced at the DNA Sequencing and Oligonucleotide Synthesis Facility of the Institute of Biochemistry and Biophysics in Warsaw, Poland.

**Results**

The aim of using perpendicular electrophoresis was to analyse the melting behaviour of given double stranded DNA fragment corresponding to the local temperature and to opitmize the conditions for an efficient separation of a given molecule in one step. Optimization means in this context the narrowing of the temperature range in which separation of bands is effective.
Table 1
Variability in codons 136, 154 and 171

<table>
<thead>
<tr>
<th>Codon</th>
<th>Alternative amino acid</th>
<th>DNA sequence</th>
</tr>
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<tbody>
<tr>
<td>136</td>
<td>Valine (Val, V)</td>
<td>GTC</td>
</tr>
<tr>
<td></td>
<td>Alanine (Ala, A)</td>
<td>GCC</td>
</tr>
<tr>
<td></td>
<td>Arginine (Arg, R)</td>
<td>CGT</td>
</tr>
<tr>
<td>154</td>
<td>Histidine (His, H)</td>
<td>CAT</td>
</tr>
<tr>
<td></td>
<td>Arginine (Arg, R)</td>
<td>CGG</td>
</tr>
<tr>
<td>171</td>
<td>Glutamine (Gln, Q)</td>
<td>CAG</td>
</tr>
<tr>
<td></td>
<td>Histidine (His, H)</td>
<td>CAT</td>
</tr>
</tbody>
</table>

Table 2
PCR primers used in TGGE analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence of the primer</th>
</tr>
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<tbody>
<tr>
<td>313 – sense</td>
<td>5’-(GC 40)GTCAAGGTGTAAGCCACAGTCAGTGG-3’</td>
</tr>
<tr>
<td>316 – anti sense</td>
<td>5’-(GC 40)GCTCCACCACTCGCTCCATTATCTTTG-3’</td>
</tr>
<tr>
<td>(GC 40)GC-clamp</td>
<td>(GC 40)GC-clamp</td>
</tr>
</tbody>
</table>

Perpendicular TGGE was first performed using PCR amplified DNA fragments from two sheep with known PrP genotype (sheep No. 3135 - AHQ/AHQ and sheep No. 117 - ARR/ARR) (Fig. 1). Based on the results of this optimization the parallel TGGE has been performed (Fig. 2).

Fig. 2 shows the distribution of PrP alleles of the selected sheep. DNA fragments having the same sequence are easily distinguishable because they migrate during electrophoresis the same distance from the starting point. In addition, the presence of single bands at slightly different locations reflects existence of different homozygotes. By analogy the presence of 4 bands inside of one lane reflects presence of different heterozygotes. The results of TGGE were confirmed by sequencing. All 20 PrP genotyped sheep were homozygous at codon 136 (AA136) (Table 3). Homozygosity in codon 154 of PrP gene was found in all 10 sheep belonging to Świarińska breed and in 5 Wrzosówka sheep. RH heterozygosity in codon 154 of PrP gene was found in the 3 remaining Wrzosówka sheep and only one sheep revealed HH homozygosity in this codon (Table 3). Analysis of codon 171 revealed more genetic variation as compared to the codons 136 and 154. The most frequently found amino acid in this codon was glutamine (Q), which occurred in the form of homozygous (QQ), as well as heterozygous allele together with histidine or arginine (QH or RQ). Only two sheep of Wrzosówka breed were homozygous (RR) in this codon. In contrary analysis of codon 171 in sheep belonging to Świarińska breed revealed homozygosity RR171 in 7 cases and heterozygosity RQ171 in the remaining 3 cases (Table 3).
Fig. 1. TGGE with a temperature gradient perpendicular to the direction of the electric field. The fragment consist of the sequence amplified using 313-sense and 316-antisense primers (Table 2). Equal volumes of PCR product containing mutations (aa) and wild type PCR product (AA) were mixed and subjected to the denaturation/renaturation process. As a result 4 different DNAs were obtained (aa, AA, Aa, aA). On the left side of the gel double stranded DNA in the form of continuous band is visible. On the right side of the gel completely denatured, single stranded DNA is visible. In the middle part of the gel 4 different curves formed by partly denatured DNA strains are visible. Partial denaturation (at the side of mismatched basis pair) delays DNA migration at given temperature. Each of the curves has different melting temperature (Tm), depending on the nucleotide sequence. Heterozygous DNAs (aA, Aa) have the lowest Tm. In contrary homozygous DNAs are more stable and have higher Tm.

Fig. 2. TGGE parallel electrophoresis of amplification products of sheep PrP gene using 313-sense and 316-antisense primers. Temperature gradient (40°C → 60°C) is same as the direction of the electric field. Lanes 2, 3, 6, 8, 9, 11- 4 bands are visible corresponding to heterozygous material. Lanes 4, 5, 10-single bands are visible corresponding to homozygous material. Lanes 1 and 12-marker. The migration distance slightly different for both hetero- as well as homozygous samples reflects differences in nucleotide sequence.
Table 3
PrP genotypes of Wrzosówka and Świąciarka breeds

<table>
<thead>
<tr>
<th>Genotyp</th>
<th>No. of Wrzosówka breed</th>
<th>No. of Świąciarka breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA_{136}RR_{154}RR_{171}</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>AA_{136}RH_{154}QH_{171}</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>AA_{136}RR_{154}RQ_{171}</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>AA_{136}RH_{154}QQ_{171}</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>AA_{136}HH_{154}QQ_{171}</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>AA_{136}RR_{154}QQ_{171}</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Discussion

This is the first preliminary study of the PrP gene polymorphism in the Wrzosówka and Świąciarka breeds of sheep. TGGE method is a powerful and sensitive technique, which can detect single point mutations in DNA/RNA molecules. For this reason TGGE can replace the more expensive sequencing method and can be used for routine screening of sequence polymorphisms in population studies. Examination of PrP gene in a group of 20 randomly selected sheep revealed lack of variation in codon 136. In contrary, the polymorphism was found in codons 154 and 171.

Among 5 alleles important in the context of susceptibility to scrapie two of them, i.e. ARH and VRQ were not present in the examined group of sheep (Table 3). VRQ encoding allele is very rare or not present in some sheep breeds like Suffolk (7, 11), Mongolian sheep (6) or Massese (9). If however, this allele appears it confers high risk of scrapie. In one scrapie-affected Scottish flock 80% of scrapie cases harboured at least one VRQ allele (14). The present study revealed high proportion of ARR encoding allele in Świąciarka breed. This PrP genotype is also frequently present in Suffolk sheep (7, 11), Massese sheep (9), Romanov sheep (5) and Valachian sheep (15). Allele ARR and AHQ are associated with resistance to scrapie, so high frequency of this alleles in some breeds of sheep can constitute basis for selective breeding programme aimed at increasing the genetic resistance to scrapie of the national sheep flock. In countries where scrapie was not yet registered such selection can diminish the risk of its outbreak.

The results of the present study confirm that TGGE method of the genotyping of the PrP gene can be useful in routine screening of the national sheep flock.

Although the presented survey was very limited (only 20 animals of two breeds were PrP genotyped) one can suspect that screening of large number of sheep can provide information on which breeds display scrapie resistant genotype. In our experiment Świąciarka breed was genetically much more resistant to scrapie (high frequency of ARR/ARR genotype) as compare to Wrzosówka (presence of Q_{171} associated with high scrapie risk) (Table 3).

One can suspect that scrapie resistant genotype of Świąciarka breed is not just accidental but can be a common feature of other primitive breeds of sheep. Świąciarka breed is present in Polish mountains since centuries in agreement with lack of evidence for scrapie in the past. This situation can change in the future in context of increased international trade and movement of animals.
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References