UNIWERSYTET TECHNOLOGICZNO-PRZYRODNICZY
IM. JANA I JĘDRZEJA ŚNIADECKICH
W BYDGOSZCZY
ZESZYTY NAUKOWE NR 255 – ZOOTECHNIKA 38 (2010) 19-26

ANALYSIS OF CAG/AAG POLYMORPHISM INCIDENCE AT CODON 171 OF THE OVINE PRION PROTEIN GENE

Agnieszka Markowska, Ewa Wiśniewska, Magdalena Szkudlarek-Kowalczyk, Sławomir Mroczkowski

University of Technology and Life Sciences in Bydgoszcz
Department of Genetics and General Animal Breeding
Faculty of Animal Breeding and Biology
Mazowiecka 28, 85-084 Bydgoszcz

The ovine *PRNP* gene is responsible for the production of cell prion protein and its polymorphism significantly affects the sheep susceptibility/resistance to scrapie. The aim of the study presented herein was to detect variation (CAG/AAG) occurring at codon 171 of the ovine prion protein gene. The test covered 241 animals from the following sheep breeds: Polish Merino, Blackheaded Mutton Sheep, Polish Mountain Sheep, Ile de France, Berrichon du Cher and Suffolk. The test itself was conducted with the application of the PCR-RFLP method and with the use of the *Mbo*I enzyme. A lysine coding triplet was found in the 171 position in case of the Blackheaded Mutton Sheep group. The XXK allele occurred in a heterozygous combination with a frequency of 0.69%, constituting 1.33% of genotypes of the analysed Blackheaded Mutton Sheep group and 0.4% of genotypes of the whole population subject to study.

Keywords: scrapie, *PRNP*, polymorphism, lysine, sheep

1. INTRODUCTION

Scrapie is a degenerative disease that affects the nervous system of sheep and goats. Scrapie was first described in 1723 in Scotland. Both scrapie and other diseases from the brain amyloidoses group has up until today remained incurable. The *PRNP* gene is responsible for coding the prion protein which in case of sheep is located within the long arm of chromosome 13, in the 13q17-q18 position [14]. Sheep have revealed polymorphisms at the following codons of the prion protein gene: 83, 85, 101, 112, 127, 136, 137, 138, 141, 142, 143, 146, 151, 154, 167, 168, 171, 172, 175, 176, 180, 189, 195, 196, 231, 237, 241 [1, 4, 7, 8, 9, 10, 13, 17, 18]. Polymorphism of the prion protein gene leads to synthesis of prion proteins of various amino acid sequences, which constitutes the cause of diversified resistance to infections and disease development. The polymorphisms occurring particularly at three codons: 136 (GCC/GTC), 154 (CGG/CAT) and 171 (CGG/CAG, CAG/CAT) have a considerable influence on the degree of sheep resistance/susceptibility to scrapie [9]. Due to this variability, five main haplotypes (hereinafter referred to as alleles) may be distinguished: $A_{136}R_{154}R_{171}$, $A_{136}R_{154}Q_{171}$,

$A_{136}H_{154}Q_{171}$, $A_{136}R_{154}H_{171}$, $V_{136}R_{154}Q_{171}$. The ARR allele determines the highest degree of sheep resistance to this disease, followed by the AHQ, ARH, ARQ alleles, with the VRQ allele determining the highest susceptibility to scrapie. The aforesaid alleles combine to form 15 genotypes which were divided into five classes of sheep resistance to scrapie, from G1 to G5 [6].

In order to detect prion protein genotypes, molecular biology methods are used, such as, e.g.: the PCR-RFLP method [10], sequencing [12], SSCP, DGGE [17]. Selection of the applicable method depends on the number of tested animals and the costs of analysis. PCR-RFLP constitutes the most frequently used method for polymorphism detection at the three most important codons: 136, 154 and 171. Another CAG/AAG single nucleotide polymorphisms (SNP) responsible for coding the lysine amino acid was discovered in the 171 triplet as a result of a study on the ovine *PRNP* gene variability [7, 10, 11]. The most frequently used enzymes enabling the CGG/CAG, CAG/CAT (R/H/Q) polymorphism detection at codon 171 are the *Bsp*HI and *Bsp*DI enzymes recognizing the CGG and CAG and CAT sequence [16]. Unfortunately, these enzymes prevent detection of the CAG/AAG polymorphism responsible for coding lysine in this position. In case of utilization of the PCR-RFLP reaction in genotyping the prion protein gene at three codons: 136, 154, 171, it is necessary to trigger an additional reaction in order to detect the AAG sequence in the 171 triplet.

Tests carried out in order to detect the CAG/AAG polymorphism were performed in several countries: Mongolia [10], Greece [5], the United States of America [11] and Italy [3]. Unfortunately, there is currently no information available on the Polish population of sheep, even though the occurrence of such single nucleotide polymorphisms (SNP) may alter the result of genotyping carried out with the use of PCR-RFLP method. It was for this reason that the purpose of the study involved detection of the CAG/AAG polymorphism in the 171 triplet, responsible for coding lysine in this position in the case of the following group of sheep: Polish Merino, Blackheaded Mutton Sheep, Ile de France, Polish Mountain Sheep, Suffolk and Berrichon du Cher, that can be found in the territory of Poland.

2. MATERIAL AND METHODS

The study covered a population of 241 sheep. The following sheep breeds were subject to testing: Polish Merino (8 males and 27 females), Blackheaded Mutton Sheep (15 males and 60 females), Polish Mountain Sheep (13 females), Ile de France (5 males and 66 females), Berrichon du Cher (5 males and 25 females) and Suffolk (3 males and 14 females). Peripheral blood of sheep collected from the jugular vein into test tubes containing K_2EDTA anticoagulant served as the biomaterial. High quality genome DNA was isolated with the use of MasterPure™ DNA Purification Kit for Blood (Epicentre Technologies). In order to detect polymorphism at the 171 codon (CAG/AAG) the PCR-RFLP technique was utilized with the use of the *Mbo*I enzyme, as per the Lühken et al. [15] methodology, with certain own modifications. The polymerase chain reactions (PCR) were performed in a volume of 25 µl. The reaction mixture contained: 1.5U Dream Taq (Fermentas) polymerase, 200 µM of each dNTP, 2.0 mM $MgCl_2$ and 10 pmol of each of the following primers: Forward 5`AACCAACATGAAGCATGTGGC3`, Reverse 5`AAGCAAGAAATGAGACACCACC3` and 100ng DNA. The thermal reaction profile consisted of: preliminary denaturation at 94°C for 90 seconds, followed by 40 cycles

covering: 94°C for 15 seconds, 60°C for 20 seconds, 72°C for 45 seconds and final synthesis at 72°C for 5 minutes. The amplified fragment had a length of 545 base pairs. In order to detect the CAG/AAG polymorphism at codon 171 of the ovine *PRNP* gene responsible for coding lysine in this position, the amplicon was subject to enzymatic hydrolysis with 3U of the *Mbo*I (Fermentas) restriction enzyme at a temperature of 37°C, for 4 hours. Enzymatic digestion products were separated in 2.5% agarose gel with 0.5 µg/µl ethidium bromide in a 1 x concentrated TBE buffer (10xTBE: 0.89M Tris, 0.89M boric acid, 0.02M EDTA, pH 8.0) at 120 V for 60 min. In case of occurrence of the AAG sequence at codon 171 the *Mbo*I restriction enzyme hydrolysed the amplicon to a 379 bp long fragment proving the incidence of lysine and to two fragments with a length of 182 bp and 197 bp, proving the incidence of another allele. Subsequently, the XXK allele occurrence frequencies were calculated for the whole group of animals covered by the study as well as for each individual sheep breed.

3. RESULTS

As a result of the performed (CAG/AAG) SNP detection in triplet 171 of the ovine *PRNP* gene it has been revealed that the XXK allele occurred in one Blackheaded Mutton Sheep female. No incidence of a lysine coding sequence at codon 171 has been identified in the case of the following sheep breeds: Polish Merino, Polish Mountain Sheep, Suffolk, Ile de France, Berrichon du Cher. The XXK allele was found in one specimen, which constituted 0.2% of alleles of the whole analysed sheep population and 0.66% of the Blackface sheep group. The genotype containing lysine occurred in a heterozygous XXK/XXX combination. Proportion of the genotype containing this allele totalled 0.4% of all genotypes within the analysed sheep population and 1.33% of the Blackheaded Mutton Sheep group. Frequency of the allele responsible for coding lysine in triplet 171 is exceptionally low within the sheep population covered by the study. Owing to its low frequency of occurrence this allele has not been categorized into any of the classes of sheep resistance to scrapie.

Figure 1 presents electrophoretic distribution of the products of *Mbo*I restriction enzyme amplicon hydrolysis reaction: M – DNA fragment length marker pUC19 (Fermentas); 1-9 – specimens without a restriction site for the *Mbo*I enzyme at codon 171, DNA fragments with the following lengths were obtained: 197 bp and 182 bp; 10 – specimen possessing a restriction site for the *Mbo*I enzyme at codon 171, DNA fragments with the following lengths were obtained: 379 bp, 197 bp and 182 bp (Fig. 1.)

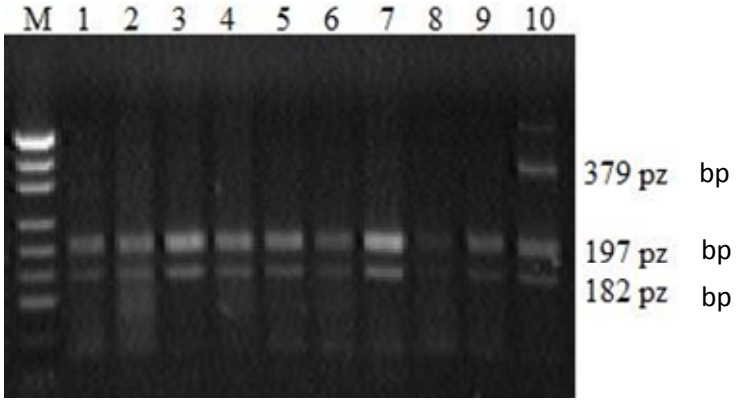

Fig. 1. Electrophoretic distribution of the products of *Mbo*I restriction enzyme amplicon hydrolysis reaction: M – DNA fragment length marker pUC19 (Fermentas); 1-9 – specimens without a restriction site for the *Mbo*I enzyme at codon 171, DNA fragments with the following lengths were obtained: 197 bp and 182 bp; 10 – specimen possessing a restriction site for the *Mbo*I enzyme at codon 171, DNA fragments with the following lengths were obtained: 379 bp, 197 bp and 182 bp

Rys. 1. Rozdział elektroforetyczny produktów rekcji hydrolizy amplikonów enzymem restrykcyjnym *Mbo*I: M – marker długości fragmentów DNA pUC19 (Ferments); 1-9 – osobniki nieposiadające miejsca restrykcyjnego dla enzymu *Mbo*I w kodonie 171, otrzymano fragmenty DNA o długości: 197 pz i 182 pz; 10 – osobnik posiadający miejsce restrykcyjne dla enzymu *Mbo*I w kodonie 171, otrzymano fragmenty DNA o długości: 379 pz, 197 pz i 182 pz

4. DISCUSSION

The genetic tests revealed a very low frequency of CAG/AAG polymorphism incidence at codon 171 of the ovine prion protein gene within the sheep population covered by the studies. Furthermore, only the XXK allele occurrence in a heterozygous combination has been confirmed. Studies carried out by researchers all around the world confirm occurrence on the ARK allele only in heterozygous combination [2, 3, 5, 10, 11]. This allele has been identified in a population of the following sheep breeds: Khalkh, Yeroo and Orkhon [10], Biellmont [2], Chios and Karagouniko [5] as well as in case of Dorper, Barbados Blackbelly, Barbados/St. Croix and Suffolk [11]. The highest frequency of the XXK allele has been noted in the case of the Biellese sheep breed in Italy [3] and amounted to 2.5%. This result was followed by dairy sheep from Greece [5] followed in turn by the Khalkh breed in Mongolia with 0.6% [10] and the Black-headed Mutton Sheep breed (own research) with 0.4%. The lowest frequency has been identified in Oklahoma, United States, at a level of 0.35% [11].

Studies carried out in Italy in 2004 covered 1207 sheep of the Biellmont breed from the Piedemount region [3]. The AAG sequence in triplet 171 was discovered in a group of 59 sheep, which amounted to 4.9% of all sheep subject to the study. The ARK allele occurred in a homozygous ARK/ARK combination in case of 2 specimens, totalling 0.2% of all genotypes. The percentage proportion of heterozygotes with the ARK allele was as follows: ARQ/ARK (n = 46) 3.8%, ARH/ARK as well as

AHQ/ARK in 4 specimens, which constituted 0.3%, VRQ/ARK in 2 specimens, totalling 0.2% and ARR/ARK in case of a single specimen, placed last in terms of percentage proportion in the frequency of genotypes within the analysed population [3].

In Greece the studies covered 216 sheep of the Chios and Karagouniko breed (Billinis, 2004). Polymorphism in triplet 171 responsible for coding lysine was discovered in case of 7 specimens in a heterozygous ARQ/ARK combination, which totalled 3.2% of the analysed population. No incidence of the ARK allele in a homozygous combination has been observed [5].

In Mongolia the studies covered 271 sheep of four breeds: Khalkh, Yeroo, Orkhon and Khangai, all inhabiting the central region of Mongolia. The ARK allele in a homozygous combination was discovered only in case of one specimen of the Khalkh breed, which amounted to a 0.6% frequency both for genotypes as well as alleles [10].

In Oklahoma, the XXK allele was discovered in case of 8 specimens of the following breeds: Dorper (n = 1), Barbados Blackbelly (n = 2), Barbados/St. Croix (n = 2) and Suffolk (n = 3) [11].

The discovery of another lysine coding mutation in triplet 171 of the *PRNP* gene initiated researches on the connection between the ARK allele and the sheep susceptibility to scrapie. The allele was found in Italy in a group of Biellmont breed specimens, both in homozygous as well as heterozygous combinations. It has been proved that the incidence of the ARK allele is related to low resistance to scrapie. It has further been discovered that the ARK allele occurred more frequently in a group of sick sheep. Due to an exceedingly low level of the ARK allele, genotypes in which it occurred have not been categorized into any of the scrapie resistance classes [2]. The same group of researchers conducted further studies in order to establish the susceptibility to scrapie revealed by sheep with a genotype containing at least one ARK allele. Due to low frequency of this allele and insufficient amount of analyses, ARK has not been categorized into any of the scrapie resistance classes [2].

Own researches as well as the studies carried out in Italy [3], Greece [5], Mongolia [10] and the United States of America [10] this polymorphism occurred with a very low frequency in case of the following sheep breed populations: Blackheaded Mutton Sheep, Biellmont, Khalkh, Yeroo, Orkhon, Dorper, Barbados Blackbelly, Barbados/St. Croix and Suffolk. The inconsiderable amount of specimens with CAG/AAG polymorphism in the lysine coding triplet 171 of the *PRNP* gene does not occur frequently enough for this allele to be categorized into any of the scrapie resistance classes. Owing to the identification of polymorphism in the lysine coding triplet 171, it is necessary to continue the studies leading to classification of the ARK allele to the relevant scrapie resistance class.

The frequency of this allele in the case of sheep populations covered by the studies is exceedingly low, that is why it has not been categorized into any of the scrapie resistance classes as per DEFRA, Great Britain.

5. CONCLUSIONS

The SNP in triplet 171 of the ovine *PRNP* gene triggering lysine coding is undetectable by means of standard DNA tests employing the PCR-RFLP method to detect the five alleles responsible for animal susceptibility to scrapie: ARR, ARQ, ARH,

AHQ, VRQ. Low frequency of occurrence of this allele within the group of six sheep breeds covered by the studies does not indicate the necessity to introduce tests enabling detection of the AAG sequence at codon 171. Standard laboratory procedures enabling detection of the five most common alleles of the prion protein seem adequate in case of the analysed breeds. It is however advisable to conduct similar studies covering other sheep breeds kept in the territory of Poland.

REFERENCES

[1] Acin C., Martin-Burriel I., Goldmann W., Lyahyai J., Monzon M., Bolea R., Smith A., Rodellar C., Badiola J.J., Zaragoza P., 2004. Prion protein gene polymorphisms in healthy and scrapie-affected Spanish sheep. J. Gen. Virol. 85, 2103–2110.

[2] Acutis P.L., Martucci F., Mazza M., Peletto S., Iulini B., Corona C., Bozzetta E., Casalone C., Caramelli M., 2006. A case of scrapie in a sheep carrying the lysine-171 allele of the prion protein gene. Arch. Virol. 151, 1875–1880.

[3] Acutis P.L., Sbaiz L., Verburg F., Riina M.V., Ru G., Moda G., Caramelli M., Bossers A., 2004. Low frequency of the scrapie resistance-associated allele and presence of lysine-171 allele of the prion protein gene in Italian Biellese ovine breed. J. Gen. Virol. 85, 3165–3172.

[4] Benkela B.F., Valleb E., Bissonnettec N., Farida A.F., 2007. Simultaneous detection of eight single nucleotide polymorphisms in the ovine prion protein gene. Mol. Cell. Probes. 21, 363–367.

[5] Billinis C., Psychas V., Leontides L., Spyrou V., Argyroudis S., Vlemmas I., Leontides S., Sklaviadis T., Papadopoulos O., 2004. Prion protein gene polymorphism in healthy and scrapie-affected sheep in Greece. J. Gen. Virol. 85, 547–554.

[6] DEFRA, 2006. In National Scrapie Plan for Great Britain.

[7] DeSilva U., Guo X., Kupfer D.M., Fernando S.C., Pillai A.T.V., Najar F.Z., So S., Fitch G.Q., Roe B.A., 2003. Allelic variants of ovine prion protein gene (PRNP) in Oklahoma sheep. Cytogenet. Genome Res. 102, 89–94.

[8] Goldmann W., Baylis M., Chichota C., Stevenson E., Hunter N., 2005. Frequencies of PrP gene haplotypes in British sheep and the implications for breeding programmes. J. Appl. Microbiol. 98, 1294–1302.

[9] Goldmann W., Houston F., Stewart P., Perucchini M., Foster J.,Hunter N., 2006. Ovine prion protein variant A136R154L168Q171 increases resistance to experimental challenge with bovine spongiform encephalopathy agent. J. Gen. Virol. 87, 3741–3745.

[10] Gombojav A., Ishiguro N., Horiuchi M., Serjmyadag D., Byambaa B., Shinagawa M., 2003. Amino acid polymorphisms of PrP gene in Mongolian sheep. J. Vet. Med. Sci. 65(1), 75–81.

[11] Guo X., Kupfer D.M., Fitch G.Q., Roe B.A., DeSilva U., 2003. Identification of a novel lysine-171 allele in the ovine prion protein (PRNP) gene. Anim. Genet. 34, 302–318.

[12] Kutzer T., Pfeiffer I., Breng B., 2002. Identification of new allelic variants in the ovine prion protein (PrP) gene. J. Anim. Breed. Genet. 119, 201–208.

[13] Lan Z., Wang Z.L., Liu Y., Zhang X., 2006. Prion protein gene (PRNP) polymorphisms in Xinjiang local sheep breeds in China. Arch. Virol. 151, 2095–2101.

[14] Lee I.Y., Westaway D., Smit A.F., Wang K., Seto J., Chen L., Acharya C., Ankener M., Baskin D., Cooper C., Yao H., Prusiner S.B., Hood L.E., 1998. Complete genomic sequence and analysis of the prion protein gene region from three mammalian species. Genome. Res. 8, 1022–1037.

[15] Lühken G., Lipsky S., Peter C., Erhardt G., 2008. Prion protein polymorphisms in autochthonous European sheep breeds in respect to scrapie eradication in affected flocks. Small Ruminant Res. 75, 43–47.

[16] Niżnikowski R., Lühken G., Strzelec E., Lipsky S., Popielarczyk D., Erhard G., 2006. Consorcium ECONOGENE PRNP gene polymorphism in 136, 154 and 171 codons in Polish sheep breeds. Med. Wet. 62, 938–941.

[17] Thorgeirsdottir S., Sigurdarson S., Thorisson H.M., Georgsson G., Palsdottir A., 1999. PrP gene polymorphism and natural scrapie in Icelandic sheep. J. Gen. Virol. 80, 2527–2534.

[18] Zhou H., Hickford J.G.H., Fang Q., 2005. Determination of alleles of the ovine PRNP gene using PCR-single-strand conformational polymorphism analysis. J. Anim. Sci. 83, 745–749.

BADANIE OBECNOŚCI POLIMORFIZMU CAG/AAG W KODONIE 171 GENU BIAŁKA PRIONOWEGO OWIEC

Streszczenie

Owczy gen *PRNP* odpowiedzialny jest za produkcję białka prionowego komórkowego prawidłowego, a jego polimorfizm ma znaczący wpływ na podatność/oporność owiec na trzęsawkę (scrapie). Celem badań była detekcja zmienności (CAG/AAG) występującej w kodonie 171 owczego genu białka prionowego. Przebadano 241 osobników ras: merynos polski, czarnogłówka, polska owca górska, ile de france, berrichon du cher oraz suffolk metodą PCR-RFLP przy użyciu enzymu *Mbo*I. W pozycji 171 wykryto triplet kodujący lizynę w grupie owiec rasy czarnogłówka. Allel XXK wystąpił w układzie heterozgotycznym z częstością 0,69%, co stanowiło 1,33% genotypów przebadanej populacji czarnogłówki oraz 0,4% genotypów całej populacji objętej badaniami.

Słowa kluczowe: trzęsawka (scrapie), *PRNP*, polimorfizm, lizyna, owce

The following study was partly financed through scholarship granted as part of the "2008/2009-ZPORR scholarships for doctoral students" project – action 2.6 of the Integrated Operational Program for Regional Development (ZPORR), project no. Z/2.04/II/2.6/20/09, with 75% financed from the funds of the European Social Fund and 25% from the Budget funds.

Artykuł zostały sfinansowany ze stypendium otrzymanego w ramach projektu „Stypendia dla doktorantów 2008/2009-ZPORR" – działania 2.6 ZPORR, nr projektu Z/2.04/II/2.6/20/09 finansowanego w 75% ze środków Europejskiego Funduszu Społecznego Unii Europejskiej oraz w 25% ze środków Budżetu Państwa.