Animal Science Papers and Reports vol. 30 (2012) no. 2, 151-158
Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Adaptation of primer extension method for monitoring of scrapie-associated genotypes in sheep *PRNP* gene

Grzegorz Smołucha[1], Artur Gurgul[2]*, Agata Piestrzyńska-Kajtoch[1], Barbara Rejduch[1]

[1] Department of Animal Cytogenetics and Molecular Genetics of the National Research Institute for Animal Production, Krakowska 1, 32-083 Balice / Cracow, Poland

[2] Laboratory of Genomics of the National Research Institute for Animal Production, Krakowska 1, 32-083 Balice / Cracow, Poland

(Received April 27, 2011; accepted February 6, 2012)

The paper describes adaptation of primer extension method for genotyping scrapie-associated codons 136, 141 and 156 in sheep *PRNP* gene. As the method failed to genotype the polymorphism in codon 171 in some samples, this polymorphism was excluded from the assay. Such genotyping problems were not observed in earlier studies describing the method and cannot be easily explained. However, full accordance of genotypes in codons 136, 141 and 156 with sequencing results was obtained and thus the method can be recommended for routine application. Simultaneously, we strongly recommend to pay special attention when using the minisequencing method for genotyping codon 171 in sheep *PRNP*.

KEY WORDS: PRNP / scrapie / sheep /SNaPshot

Scrapie, the fatal neurodegenerative disease of small ruminants belongs to the group of transmissible spongiform encephalopathies (TSEs). The common feature of TSEs is deposition in the central nervous system of pathogenic prions (PrP^{Sc}), which are isoforms of cellular prion proteins (PrP^{C}). The Molecular basis of TSE pathogenesis is refolding of C-terminal α-helical domains of PrP^{C} into β-sheet

*Corresponding author: agurgul@izoo.krakow.pl

structures and aggregation of misfolded protein in the brain, *medulla oblongata* and other organs [Prusiner 1998, Bossers *et al.* 2000]. The pathological folding leads to changes in physico-chemical properties of prion protein [DeArmond and Prusiner 1995, Lau *et al.* 2007].

PrP^C is encoded by autosomal, highly interspecies-conserved prion protein gene *(PRNP)* [Groschup *et al.* 1997] *PRNP* is expressed in most tissues, but its highest expression was observed in the central nervous system and in the immunological system [Horiuchi *et al.* 2002, Wierzbicka and Deptuła 2008]. Polymorphism of *PRNP* open reading frame (ORF) resulting in amino acid substitutions was found to be associated with resistance/susceptibility of sheep to classical scrapie and it was shown that variations of PrP amino acid sequence (arising from nucleotide substitutions at codons 136, 154 and 171) affects conformational stability of prion protein [Bayls and Goldmann 2004, Goldmann *et al.* 2006; Niznikowski *et al.* 2006].

Based on genetic profile of PrP^C, sheep were classified into five classical scrapie risk groups. The first group spans sheep carrying genotypes associated with lowest scrapie risk: $A_{136}R_{154}R_{171}$/ARR, while the fifth spans genotypes most commonly observed among affected animals: AHQ/VRQ, ARQ/ VRQ, ARH/VRQ and VRQ/ VRQ [Tongue *et al.* 2004].

In 1998 in Norway an atypical variant of ovine scrapie was detected. After that, more atypical scrapie cases were identified in sheep and goat populations from different European countries. Association studies have shown that allele $A_{136}F_{141}R_{154}Q_{171}$ (ARQ with phenylalanine in codon 141 instead of leucine) is associated with an increased atypical scrapie susceptibility and a substitution of arginine by histidine in codon 154 may be related to increased atypical scrapie risk [Moum *et al.* 2005, Fediaevsky *et al.* 2008].

Because selection to increase the frequency of scrapie resistance-associated genotypes is strongly recommended as a prevention tool against scrapie, efforts were made in many European countries to establish genetic structure of sheep from flocks with high production value. In Poland in the years 2005-2009, at least 5681 sheep were tested for scrapie-associated genotypes [Piestrzyńska-Kajtoch *et al.* 2009]. Such a large number of tests is labour- and capital-consuming and requires the use of high throughput genotyping methods. To date at the National Research Institute for Animal Production, a RFLP method with BspHI enzyme was used to establish genotypes in codons 136 and 154 and a Real Time PCR Allelic Discrimination Assay for genotyping codon 171 [Garcia-Crespo *et al.* 2004]. Such complicated procedure is time-consuming and makes the assay susceptible to genotyping errors. In the last few years atypical scrapie cases were diagnosed in Polish sheep [Polak *et al.* 2010]. This encouraged including codon 141 of *PRNP* ORF in routine population genotyping.

Here we adapted and developed a previously published method [Vaccari *et al.* 2004] for genotyping sheep PrP codons 136, 154 and 171 based on SNaPshot Multiplex system (APPLIED BIOSYSTEMS, USA), which represents adaptation of primer extension technique. As some genotyping inconsistencies were found in codon

171 when compared to the sequencing results, the codon was excluded from the assay. Based on that experience, we strongly recommend to give special attention when genotyping codon 171 in sheep *PRNP* using SNaPshot-based genotyping assays. Additionally, we successfully included codon 141 into the assay.

Material and methods

Genomic DNA extracted from whole blood sample of 60 female sheep (*Ovis aries*) of five breeds was investigated. For each breed (Świniarka, Polish Mountain, Ile de France, Wielkopolska and Polish Merino) 11 – 13 samples were analysed.

To confirm primer extension technique reliability, *PRNP* genotypes were established by ORF sequencing, and sheep with known genotypes in codons 136, 141, 154 and 171 were studied. Additionally, 30 samples coming from international comparison tests organized by International Society of Animal Genetics (2010) and Veterinary Laboratories Agency (2011) were included into analysis.

DNA was isolated using The Wizard© Genomic DNA Purification Kit (PROMEGA). Amplification and sequencing of 850 bp spanning whole *PRNP* ORF was performed with primers – F: 5'-TTGATGCTGACACCCTCTTT-3' and R: 5'-GGCAAAGATTAAGAAGATAATGAAA-3'.

PCR reaction was conducted in 25 µl volume containing: 11 µl of PCR-grade water, 2.5 µl of PCR buffer with 15 mM $MgCl_2$ (QIAGEN), 5 µl of Q-Solution (5x; QIAGEN), 3 µl of 10 mM dNTPs (APPLIED BIOSYSTEMS), 0.25 µl of primer mix (each 100 pmol/µl), 0.25 µl of HotStartTaq DNA polymerase (5 U/µl QIAGEN) and 2 µl of DNA isolate. PCR thermal programme was: 15 min of initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 60 s and primer extension at 72°C for 120 s. Final step of elongation was performed at 72°C for 10 min.

PCR products were purified with ExoSAP-IT enzyme mixture (USB CORPORATION) and sequenced from both complementary strands using BigDye Terminator v3.1 Cycle Sequencing Kit (APPLIED BIOSYSTEMS). The sequencing products were purified using BigDye XTerminator Purification Kit (APPLIED BIOSYSTEMS) and sequence was red by capillary electrophoresis on 3130xl Genetic Analyzer (APPLIED BIOSYSTEMS).

Primer extension reaction

In single-base extension (SBE) technique the *PRNP* ORF fragment from nucleotide 287-613 (spanning all studied polymorphic sites) was amplified using primers described by Vaccari *et al.* [2004]. PCR product was tested for specificity and relative yield on 2% agarose gel. PCR amplification was carried out in a 25 µl reaction volume containing 13.3 µl of PCR-grade water, 2.5 µl of PCR buffer with 15 mM $MgCl_2$, 5 µl of Q-Solution (5x; QIAGEN), 1 µl of 10 mM dNTPs (APPLIED BIOSYSTEMS), 1 µl of primer mix (each 10 pmol/µl), 0.2 µl of HotStartTaq DNA polymerase (5 U/µl;

QIAGEN) and 2 µl of genomic DNA isolate. PCR thermal conditions were as follows: 15 min of initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 40 s, annealing at 57°C for 30 s, primer extension at 72°C for 45 s. Final extension step was performed at 72°C for 10 min. PCR products were purified from unincorporated dNTP's and primers using ExoSAP-IT (USB CORPORATION). 2.5 µl ExoSap and 5 µl PCR product were incubated at 37°C for 30 min. and at 80°C for 15 min.

SNaPshot reaction was carried out in 10 µl volume, containing 3 µl of purified PCR product, 3 µl of SNaPshot reaction mix, 2 µl of SBE primer mix (0.2 µM each) and 2 µl of PCR-grade water. Thermal programme comprised 25 cycles of incubations: 96°C for 10s, 54°C for 7 s and 60°C for 30 s. Reaction product was cleaned up from unincorporated [F] ddNTP by addition of 1 unit of SAP enzyme and incubation for 60 min at 37°C and 15 min at 75°C. Purified products were electrophoresed using 3130xl Genetic Analyzer, Gene Scan LIZ 120 Size Standard and POP-7 polymer (APPLIED BIOSYSTEMS).

The SBE primers allowed for polymorphism detection in codons 136 and 154 (as described by Vaccari *et al.* [2004]) and in codon 141 of *PRNP* ORF (Tab. 1). As some genotyping inconsistency was found in codon 171 when compared to sequencing results, both primers described by Vaccari *et al.* [2004] (allowing for genotyping SNPs at nucleotides 512 and 513) were excluded from analysis.

Results and discussion

The method presented here allows for genotyping SNPs in codons 136, 141 and 154 in sheep *PRNP* ORF and is based on minisequencing, which uses single-base extension (SBE) technique and capillary electrophoresis. In the method, SBE primers bind just before polymorphic site in preamplified template and are elongated by one dideoxynucleotide, which is fluorescent- labelled and defines the allele ([F]ddNTP). Each primer migrates separately in the electrophoresis due to the differences in the primers length. The differences in primer lengths arise from addition during the synthesis of poly-T tails of different length to the 5' end of each primer (Tab. 1). The method is suitable for different capillary electrophoresis-based genotyping platforms

Table 1. The SBE primers sequences

Name	Sequence 5`-3`	Length (bp)	Orientation F/R	Codon
p407*	$(T)_{15}$ GGTGGCTACATGCTGGGAAGT G	37	F	136
p421	$(T)_{22}$GGTCCTCATAGTCATTGCCAAAATGTATAA	52	R	141
p461*	$(T)_{9}$ GGG GTAACG GTACATGTTTTCA	31	R	154
p512^{E}*	CAAGTGTACTACAGACCAGTGGATC	25	F	171
p513^{E}*	$(T)_{19}$GCACAAAGTTGTTCTGGTTACTATA	44	R	171

*Described by Vaccari *et al*. [2004].
^{E}Excluded from the assay.

and available protocols are simple to adapt in particularly every molecular laboratory. Moreover, the technique is flexible for partial automatization and results can be easily processed using simple informatics tools.

The primary aim of the study was to adapt the previously published method [Vaccari *et al.* 2004] for genotyping sheep *PRNP* codons 136, 154 and 171 in the National Research Institute for Animal Production and extend it by including atypical scrapie-associated codon 141 into the assay. Unexpectedly, some genotyping inconsistencies (in about 5% of animals) occurred when genotyping codon 171. In this codon two polymorphic sites were identified by Bayls and Goldmann [2004] and Garcia-Crespo *et al.* [2004]. First SNP is located in nucleotide 512 (A→G) and results in Q to R amino acid substitution. Another SNP is located in nucleotide position 513 (G→C or T) and results in R or Q to H substitution in amino acid position 171. To genotype this codon correctly with primer extension method two SBE primers are needed designed in opposite directions. One terminates just before nucleotide 512 (171a), while the other ends one base adjacent to nucleotide 513 (171b) on the reverse

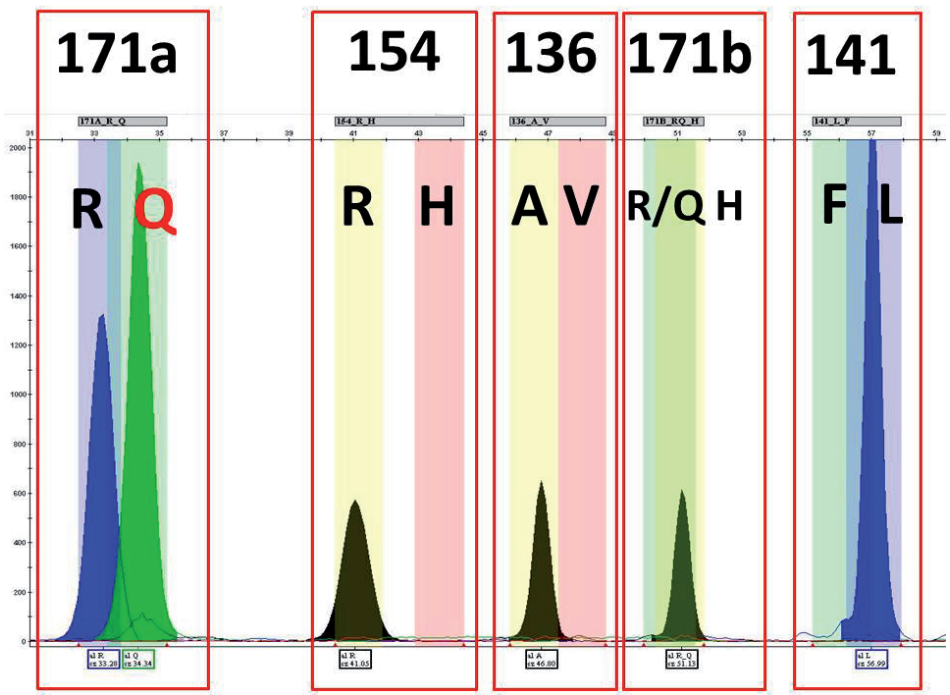

Fig. 1. Exemplary chromatogram showing incorrect results of genotyping of sample with genotype ALRR/ALRR in codons 136, 141, 154 and 171.
On the chromatogram each marker was signed in black. In marker 171a (resulting from extension of primer for genotyping nucleotide 512 in codon 171) additional peak (green) is visible and represents 171Q allele. True genotype of studied sample in codon 171 was R/R, which was confirmed in ISAG comparison test.

strand and is elongated by one base in the reverse direction. As primer used for genotyping SNP 513 (G→C or T) seemed to work well and correctly identified few studied cases of 171H allele, primer designed for genotyping SNP 512 (A→G), in about 5% of samples, gave genotypes inconsistent with traditional sequencing-based results. When using SNaPshot method, on average, we obtained incorrect genotypes in codon 171 in one per 15-20 animals. Despite high quality data (low background and unambiguous peaks) we observed both missing or additional R or Q alleles (Fig. 1). Additional peaks seemed not to result from template (PCR product migration) or undigested PCR primers as they were of appropriate size and appeared constantly in different reaction mixes and in each technical reaction repeat. PCR template was also electrophoresed separately from primers to ensure that migrating DNA fragments do not give additional peaks.

Different reaction mixtures (primer, reaction mix, PCR template concentrations) and different thermal conditions were used to calibrate the method. However, for unknown reason genotyping inconsistency persisted even when primers for genotyping codon 171 were used separately from others described in the method. Unfortunately, we cannot explain the mechanism of the phenomenon described. Such inconsistency was not found earlier among the published data concerning the method [Vaccari *et al.* 2004]. Facing the above we suggest using another method for genotyping codon 171 or paying special attention when including it in the SNaPshot-based analysis.

Due to that observation, we decided to exclude codon 171 from the assay and to design the method for genotyping codons 136, 141 and 154 in sheep *PRNP*. Nevertheless, this method is in advantage to previously used RFLP technique [Garcia-Crespo *et al.* 2004], because it gives reliable results, allows genotyping of codon 141 in the same reaction mixture and does not require agarose gels usage. We did not redesign the primers for genotyping codon 171, because primer binding site in this technique is not arbitral and has to precede polymorphism.

By using modified primer extension technique for genotyping codons 136, 141 and 154 we genotyped 60 sheep of 5 breeds and found full consistency with the results obtained with sequencing analysis. The studied group of animals represented all common *PRNP* variants as well as some rare variants including Ll41F genotype. Additionally, genotyping correctness was confirmed by results obtained from two international comparison tests involving *PRNP* polymorphism analysis in 30 animals.

GeneMapper software allowed to design specific panel of markers, in which marker range was marked and allele-corresponding bins were named in the way allowing for direct amino acid variation reading from nucleotide-based data (Fig. 2). As some primers used (codons 141 and 154) were designed in reverse direction, alleles were renamed to represent appropriate (forward) PrP sequence. Designed primer for genotyping codon 141 worked well, gave strong signal and migrated separately in the final method as well as in mixture with primers for genotyping codon 171.

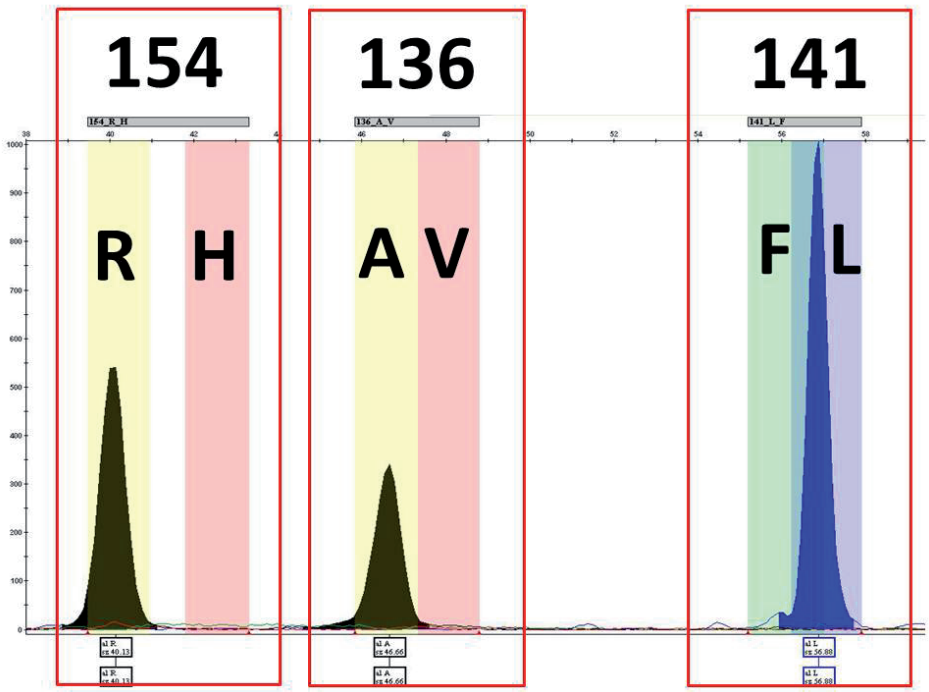

Fig. 2. Exemplary chromatogram showing results of genotyping codons 136, 141 and 154. Analysed sample genotype was ALR/ALR.

The method presented here gives precise results with all analysed data for codons 136, 141 and 154 and can be considered as reliable. Such an analysis takes about 1-2 working days with hands-on for about 2-3 hours per 96 samples. This gives advantage in comparison to RFLP-based methods [Garcia-Crespo *et al.* 2004], which are labour-consuming and results obtained cannot be directly digitalized.

In the early stage of the study when using SNaPshot system for genotyping codon 171 we found inconsistent genotypes compared to the sequencing results. Accordingly, we strongly recommend testing the method on tens of samples of known genotypes before including codon 171 into SNaPshot-based analysis methods.

REFERENCES

1. BOSSERS A., VRIES R., SMITS M., 2000 – Susceptibility of Sheep for Scrapie as Assessed by In Vitro Conversion of Nine Naturally Occurring Variants of PrP. ***Journal of Virology*** 74, 1407-1414.
2. DEARMOND S. J., PRUSINER S. B., 1995 – Etiology and Pathogenesis of Prion Diseases. ***American Journal of Pathology*** 146, 785-811.
3. FEDIAEVSKY A., TONGUE S., NÖREMARK M., CALAVAS C., RU G., HOPP P., 2008 – A descriptive study of the prevalence of atypical and classical scrapie in sheep in 20 European countries. ***BMC Veterinary Research*** 4, 19.

4. GARCIA-CRESPO D., OPORTO B., GOMEZ N., NAGORE D., BENEDICTO L., JUSTE R.A., HURTADO A., 2004 – PrP polymorphisms in Basque sheep breeds determined by PCR-restriction fragment length polymorphism and real-time PCR. ***Veterinary Record*** 154, 717-722.
5. GOLDMANN W., HOUSTON F., STEWART P., PERUCCHINI M., FOSTER J., HUNTER N., 2006 – Ovine prion protein variant A136 R154 L168 Q171 increases resistance to experimental challenge with bovine spongiform encephalopathy agent. ***Journal of General Virology*** 87, 3741-3745.
6. GROSCHUP M., HARMEYER S., PFAFF E., 1997 – Antigenic features of prion proteins of sheep and other mammalian species. ***Journal of Immunological Methods*** 207, 89-101.
7. HORIUCHI M., NEMOTO T., ISHIGURO N., FURUOKA H., MOHRI S., SHINAGAWA M., 2002 – Biological and Biochemical Characterization of Sheep Scrapie in Japan. ***Journal of Clinical Microbiology*** 40, 3421-3426.
8. LAU A., YAM A. Y., MICHELITSCH M., WANG X., GAO C., GOODSON R. J., SHIMIZU R., TIMOTEO G., HALL J., MEDINA-SELBY A., COIT D., MCCOIN C., PHELPS B., WU P., HU C., CHIEN D., PERETZ D., 2007 – Characterization of prion protein (PrP)-derived peptides that discriminate full-length PrPSc from PrPC. ***Proceedings of the National Academy of Sciences of the USA*** 104, 11551-11556.
9. PIESTRZYŃSKA-KAJTOCH A., OCZKOWICZ M., NATONEK-WIŚNIEWSKA M., PIÓRKOWSKA K., KAWECKA M., KOZUBSKA-SOBOCIŃSKA A., KNAPIK J., KRUPIŃSKI J., REJDUCH B., 2009 – International Scientific Conference: Transmissible Spongiform Encephalopathies in Farm Animals – Diagnosis and Prevention. Balice, Poland, 28-29 September, Book of Abstracts p. 57.
10. POLAK M.P, LARSKA M., LANGEVELD J.P., BUSCHMANN A., GROSCHUP M.H., ZMUDZINSKI J.F., 2010 – Diagnosis of the first cases of scrapie in Poland. ***Veterinary Journal*** 186, 47-52.
11. TONGUE S.C., WILESMITH J.W., COOK C.J., 2004 – Frequencies of prion protein (PrP) genotypes and distribution of ages in 15 scrapie-affected flocks in Great Britain. ***Veterinary Record*** 154, 9-16. Erratum in: Vet. Rec. 154, 116.
12. MOUM T., OLSAKER I., HOPP P., MOLDAL T., VALHEIM M., MOUM T., BENESTAD S.L., 2005 – Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases. ***Journal of General Virology*** 86, 231-235.
13. WIERZBICKA A., DEPTUŁA W., 2008 – Rola układu odpornościowego w patogenezie chorób prionowych. (The role of the immune system in the pathogenesis of prion diseases). In Polish with Emglish summary. **Postępy Higieny i Medycyny Doświadczalnej** 62, 166-173.