THE PRIMER EXTENSION TECHNIQUE FOR THE POLYMORPHISM DETECTION AT OVINE PRN-P LOCUS

TEHNICA PRIMER EXTENSION PENTRU DETERMINAREA POLIMORFISMELOR DE LA LOCUSUL PRN-P LA OVINE

COSIER VIORICA*, VLAIC A*, PADEANU I **, DARABAN S*, VOIA S.*, CATOI C*, CONSTANTINESCU R*, VICOVAN G ***,

** Faculty of Animal Sciences and Biotechnologies, Cluj-Napoca, România*
*** Faculty of Animal Sciences and Biotechnologies, Timişoara, România*
**** ICDOC-Palas, România*

Scrapie is a prionic illness with endemic character in many parts of the glob, and the control measures is difficult to apply because of the long incubation period, the lack of the preclinical manifestation and the existing tests for diagnostic in living animals. The Ppn-p locus is polymorphic with known variability at codon 136, 154, 171, which are associated with different sensibility in experimental and natural spongiform encephalopaties. General the possible combinations of the 5 amino acids encoded by the 3 different codons will determine the existence of 15 possible genotypes. To put in evidence those polymorphisms at the ovine Prn-p locus, several methods are developed but the most accurate assay is the direct sequencing of the gene and the primer extension technique. The purpose of this study was to determine the genotypes at Prp locus in 123 male of Tsurcana breed, Hateg ecotype, using primer extension technique (ABI 3130xl Genetic Analyzer) and to establish the risk groups of the susceptibility at scrapie disease.
Key words*: scrapie, prion, Prnp locus, primer extension, TSE*

Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of fatal neurodegenerative diseases including sheep and goat scrapie, bovine spongiform encephalopathy (BSE), and Creutzfeld-Jakob disease (CJD) in humans. The pathology of such diseases is characterized by vacuolation, neuronal loss and glial cell activation and proliferation. The pathological hallmark of TSE is the accumulation of an abnormal protein, named Prp-Sc, which is formed from the normal isoform (Prp-C). Prions are transmissible particles that are devoid of nucleic acid and seem to be composed exclusively of a modified protein (Prp-Sc).The normal, cellular PrP (PrP-C) is converted into PrP-Sc throught a posttranslational process during which it acquires a high B-sheet content. The presence of Prp-Sc is considered a marker for TSEs. Polymorphisms in the prion protein (PrP) gene are associated with different phenotypic expression of

transmissible spongiform encephalopathies in animals and humans. In sheep, at least 10 different mutually exclusive polymorphism are present in PrnP gene. The polymorphism in codons 136, 154, 171 occur frequently, while the polymorphism in codons 112, 137, 211 are rare. The polymorphism at codon 136 154 and 171 are associated with different sensibility at natural and experimental spongiform encephalopathies. General the possible combination at the 5 amino acids encoded by the 3 different codons will determine the existence of the 15 possible genotypes at the PrP locus (Gabriele Vaccari et al 2006. R. Renaville et.al. 2002).

-ARR/ARR-sheep very resistant at scrapie

-ARR/AHQ, ARR/ARH, ARR/ARQ-sheep genetic resistant to scrapie but they need a special attention for using in selection programs

-ARQ/ARH, ARQ/AHQ, AHQ/AHQ, ARH/ARH, AHQ/ARH, ARQ/ARQ-sheep with lower genetic to scrapie

-ARR/VRQ-sheep sensitive to scrapie

-AHQ/VRQ, ARH/VRQ, ARQ/VRQ, VRQ/VRQ-sheep very sensitive to scrapie

Materials and Methods

The selection of genetically resistant sheep populations represents the basis of the recent strategies against ovine TSE in the European Union. In order to accomplish this goal several methods are used, including PCR-RFLP, DGGE analysis, ARMS, Taq-Man –MGB assay, reverse hybridisation; mass spectrometry, sequencing and primer extension technique. (Van Poucke et. al., 2005)

The most accuracy methods are based on sequencing, and in this study we use the primer extension technique. In order to perform the primer extension technique for genotypization at PrnP locus is necessary to follow some steps:

- DNA extraction from the blood samples
- determination of the DNA quality and quantity
- template amplification
- **.**purification of the PCR products
- Primer extension technique
- performing the capillarity electrophoresis to determine the polymorphism
- the analysis of results for electrophoretic profiles with GeneMapper^{R} software

DNA preparation from blood samples:

Whole blood samples from 123 male sheep (Tsurcana breed, Hateg ecotype) were collected in tubes containing K3- EDTA and stored at -20^{o}C. The selection of biologic material was performed using the register of official control of productions in the aim of including these animals in the national plan for selection against scrapie disease.

The DNA used in sequencing must be superior qualitative, free from proteic or RNA contaminants.

The DNA from blood was extracted using MagnaPure LC DNA Isolation Kit (Roche). The quality and quantity of DNA was detected spectrophotometricaly using NanoDrop ND 1000 instrument.

Template amplification:

In this stage with the PCR technique is amplified partial PrP coding region. PCR amplification was carried out in a 25 µl reaction volume containing 2 µl of DNA (40-100 ng/ µl) 0,3 µl Taq polymerase (Promega), 2,5 µl PCR buffer (Promega), 1 µl dNTPmix and 2,5 µl Primer preSnaPshot mix.

The PCR amplification was performed in a DNA thermocycler (Mastercycler Eppendorf) and the cycles were: 1 x 3 minutes at 92^{o}C; 35x 60 seconds at 95^{o}C, 30 seconds la 62 ^{o}C, 30 seconds la 72^{o}C, and a final final extension cycle at 72^{o}C

The PCR product purification: High Pure purification kit (Roche):

This operation need to be performed for move away the primers, unincorporated dNTP and the inhibitors.

Primer extension technique:

They are several stages, which must be following in order to perform the genetic analysis for scrapie sensibility. The mix for the reaction is composed with SnaPshot Multiplex Ready Reaction mix (Applied Biosystem) - 5 µl, Primers SnaPshot Mix – 1 µl, H_2O – 1 µl, and 2µl of the previously purified PCR product. The primer extension reaction cycles are: 25 x 10 seconds at 96 ^{o} C, 5 seconds at 50 ^{o} C, 30 seconds at 60 ^{o} C. This reaction consist of a single nucleotide extension of primers complementary to the target DNA and adjacent to the polymorphic sites of interest. During this step, the primers incorporate fluorescently labelled dideoxynucleotides presented in the SNaPshot chemistry. Primers are designed with tails of varying lengths at the 5` end and their identification by size differences is performed in a capillary electrophoresis.

The presence of unincorporated dNTPs into the primer extension reaction was avoided by dephosphorylating deoxyribonucleotides with calf intestinal alkaline phosphatase.

Capillarity electrophoresis – 3130 xl Genetic Analyzer (Applied Biosystem)

One microliter of the reaction mixture was incubated for 5 minutes at 95^{o}C with 11,5 µl formamide and 0,5 µl Gene-Scan Lize size standard (Applied Biosystem). After capillarity electrophoresis, the electropherogrames were analyzed by GeneScan 3.1. Software (Applied Biosystem)

The analysis of results and data interpretation

The polymorphism in codons 136,154 and 171, is detected in electropherograms in this way:

If in position 136 (near position 25 of the molecular marker size) is present a black peak we have the AA genotype, a black peak and a red peak are meaning that we have AV genotype, and in case we have a single red peak the genotype is VV.

The polymorphism in the position 154 is detected depend of the blue or green color (position 35 of the molecular marker size) and here we have the genotype RR, RH, or HH.

The determination of the polymorphism in position 171 is related to the polymorphism in the position 154. Here we have 2 different situations: (1) if in between the position 35 and 50 of the molecular marker, we have a single peak green the genotypes are:

-a single blue peak, the genotype is QQ

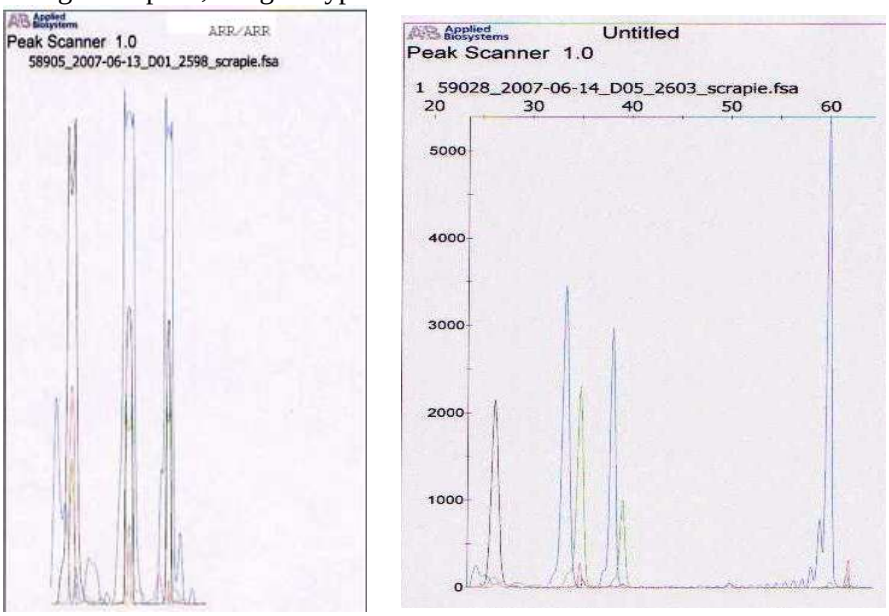
-a single red peak, the genotype is HH

-a red peak and a blue peak, the genotype is QH

If between the position 35 and 50 of the molecular marker, we have a blue peak and a green peak the genotypes are:

-a single blue peak, the genotype is RQ

-a single red peak, the genotype is RH



(a) (b)

Fig. 1 – Electropherogrames of ARR/ARR genotype (a) and ARR/AHQ (b)

Results and Discussions

After the genotypization at Prn-P locus of the 123 male sheeps of the Tsurcana breed – Hateg ecotype, from Hunedoara region, we reach at the follow conclusion:

-18 male had the ARR/ARR genotype, which is the genotype related to the highest resistance to scrapie and they represent 14, 63%.

-40 male (32,52%) had the genotypes ARR/AHQ, ARR/ARH, ARR/ARQ which are the genotype correlated with resistance to scrapie, but those require a particular attention to be use in selection programs

-33,33% (41 individues) had the genotypes ARQ/ARH, ARQ/AHQ, AHQ/AHQ, ARH/ARH ,AHQ/ARH, ARQ/ARQ, which are the genotype correlated with lower resistance to scrapie

-7 male had the ARR/VRQ, genotypes and those male are sensitive to scrapie and they represent 5, 69%

-17 male (13,82%) have been found with genotypes related with lower resistance to scrapie: AHQ/VRQ, ARH/VRQ, ARQ/VRQ ,VRQ/VRQ, and is recommended to be sacrifice.

Conclusions

The recent EU legislation identifies the selection of genetically resistant sheep as the backbone of the strategy for prevention and control the ovine TSE in Europe. The breeding programmes of sheep to select resistant genotypes with the aim of minimising the risk of the TSE or eradicate the disease from sheep populations are initiated in some European countries and will be implemented in the next future in all EU countries.

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