EVALUATION OF A NEW TEST FOR GENOTYPING CODONS 136-154-171 OF THE OVINE PRION PROTEIN (*PRNP*) GENE THROUGH REVERSE HYBRIDIZATION

Evaluatie van een nieuwe genotyperingstest voor de codons 136-154-171 van het schapenprionproteïne (PRNP) gen met behulp van "Reverse Hybridization"

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ABSTRACT

A new test for genotyping codons 136-154-171 of the ovine prion protein (*PRNP*) gene via the detection of mutations of the PrP gene through reverse hybridization has been evaluated on non-coagulated blood and brain tissue. A total of 100 sheep blood samples and 28 brain tissue samples were tested. These samples were also analyzed with denaturating gradient gel electrophoresis and real-time polymerase chain reaction to confirm the results obtained via the present test. The results obtained via these three tests corresponded perfectly. The paper briefly describes the use of the new test.

SAMENVATTING

Een nieuwe genotyperingstest voor de condons 136-154-171 van het schapenprionproteïne (PRNP) gen via detectie van mutaties in het PrP-gen met behulp van “reverse hybridization” werd geëvalueerd op niet-gestold bloed en hersenweefsel. In totaal werden 100 bloedstalen en 28 hersenstalen onderzocht. Al deze stalen werden daarna geconfirmeerd met “denaturating gradient gel electrophoresis” en “real-time polymerase chain reaction”. De resultaten van deze drie testen kwamen zeer goed overeen. In dit artikel wordt kort het gebruik van deze nieuwe genotyperingstest besproken.

INTRODUCTION

Scrapie is the oldest known transmissible spongiform encephalopathy (TSE), having been described in literature over the past two and a half centuries. It is a fatal neurodegenerative disease that occurs naturally in sheep and goats. In terms of it etiology, scrapie is currently considered to be an infectious disease with horizontally contagious maternal transmission in which the host genetic factors play a central role (Dickinson *et al.* 1974, Hunter *et al.* 1993, Belt *et al.* 1995, Smits *et al.* 1997). The natural incidence of scrapie in sheep is associated with prion protein gene polymorphism (Hunter, 1997), in particular regarding the codons 136, 154, 171.

In several breeds, scrapie susceptibility is greatly enhanced by a valine (V) substitution for alanine (A) at codon 136. On the contrary, in all breeds studied, an amino acid change from glutamine(Q) to arginine (R) at codon 171 in the PrP gene renders the animal more resistant to natural scrapie (Goldmann *et al.* 1994). The association between scrapie susceptibility and polymorphism at codon 154 is currently unclear, but there is a possibility that histidine (H) at codon 154 may offer protection from scrapie in some breeds of sheep (Elsen *et al.* 1999, Thorgeirsdottir *et al.* 1999, Tranulis *et al.* 1999).

The genotyping of sheep has become very important for the TSE eradication strategy and the breeding program aimed at developing TSE resistance (Common Decision 1803/2002).

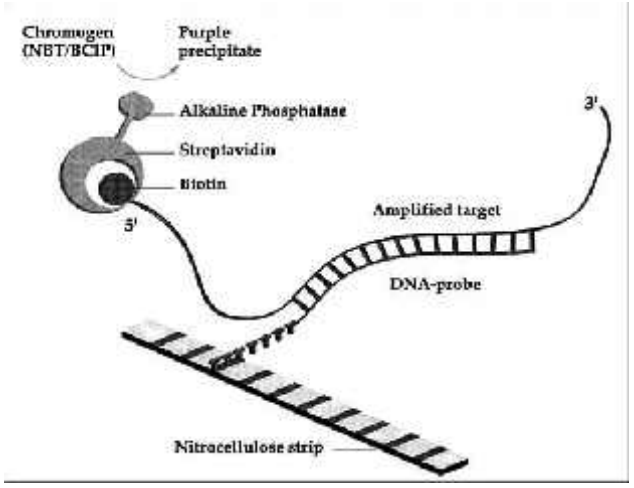

Figure 1. Hybridization of biotinilated ampliconson strips coated with allele-specific probes.

The TSE Regulation 999/2001 requires active surveillance of sheep and goats by one of the five approved rapid screening tests for TSE. The same regulation requires the PrP genotype of these TSE positive ruminants to be determined if the initial positive results are confirmed. Additionally, screening for the PrP genotype distribution of the different breeds in the EU Member States has also been suggested with a view to setting up a global breeding scheme with the principal goal of eliminating TSE in the European sheep population (Common Decision 1803/2002).

For these reasons, the Member States have had to install technologies in order to comply with this regulation. For the moment, different techniques are currently available, each based on a different approach, such as Restriction Fragment Length Polymorphism (RFLP) (Hunter *et al.*, 1993), Allele Specific Amplification (ASA) (Belt *et al.*, 1995), oligo hybridization (Ishiguro *et al.*, 1993), and denaturing gradient gel electrophoresis (DGGE) (Belt *et al.*, 1995). Many of the techniques are rather complex, laborious and time consuming, and each requires a very specific kind of expertise. This means that there is an ongoing need for more rapid tests based on new technologies to enable the reliable, fast and easy screening of the PrP gene.

Recently, genotyping via real-time polymerase chain reaction (Fontaine *et al.*, in preparation) has also become possible. The purpose of this paper is to describe the test results of the newly developed "Ovine PrP Gene Test" (REF AC019) of Nuclear Laser Medicine (NLM) (Milan, Italy) on non-coagulated blood and brain tissue. The technique, which is based on reverse hybridization, appears to be competitive with other tests available for the genotyping of sheep.

MATERIALS AND METHODS

The test consists of three steps:

1/ DNA isolation from non-coagulated blood (EDTA or citrate as anticoagulant) through a column DNA purification system.

2/ Amplification of the relevant ovine prion protein (PrP) gene sequences in the target PrP region with biotinylated primers. The amplification products consist of 224 bp.

3/ Hybridization of biotinylated amplicons on strips coated with allele-specific probes (about 20 base-pairs). The hybrids are further revealed through streptavidin conjugated with alkaline phosphatase and an appropriate substrate.

The test is available as a kit, with all the necessary buffers and solutions (almost ready to use).

The principle of the test is explained in Figure 1. The end result is a stained strip. A Decoder Table (Figure 2) is used to read the strip. For each polymorphic position (i.e. codon 136, 154 and 171), one of the three following reaction models can be obtained: (1) only wild type (WT) line, (2) wild type and mutated (MUT) lines, or (3) only mutated line. These three models correspond respectively with a homozygous wild type genotype, a heterozygous genotype and a homozygous mutated genotype.

For example: the A136V WT mark corresponds with A, while A136V MUT corresponds with V. One stained band on the tested strip of A136V WT means AA_{136} (homozygous WT), while the stained A136V MUT band means VV_{136} (homozygous MUT). Two (equally) stained bands, one being A136V WT and one being A136 MUT, mean AV_{136} (heterozygous). The same applies for the 154 codon. The 171 codon has three bands, one for Q (wild type), a second for R (mutated) and a third for H (mutated). $A_{136}R_{154}Q_{171}$ is considered to be the wild type combination.

This test was originally developed for application with non-coagulated blood, though the authors also used this test on neural tissue (25 mg ± 1 mg) and compared the results with the results of the non-coagulated blood of the same sheep. For this last

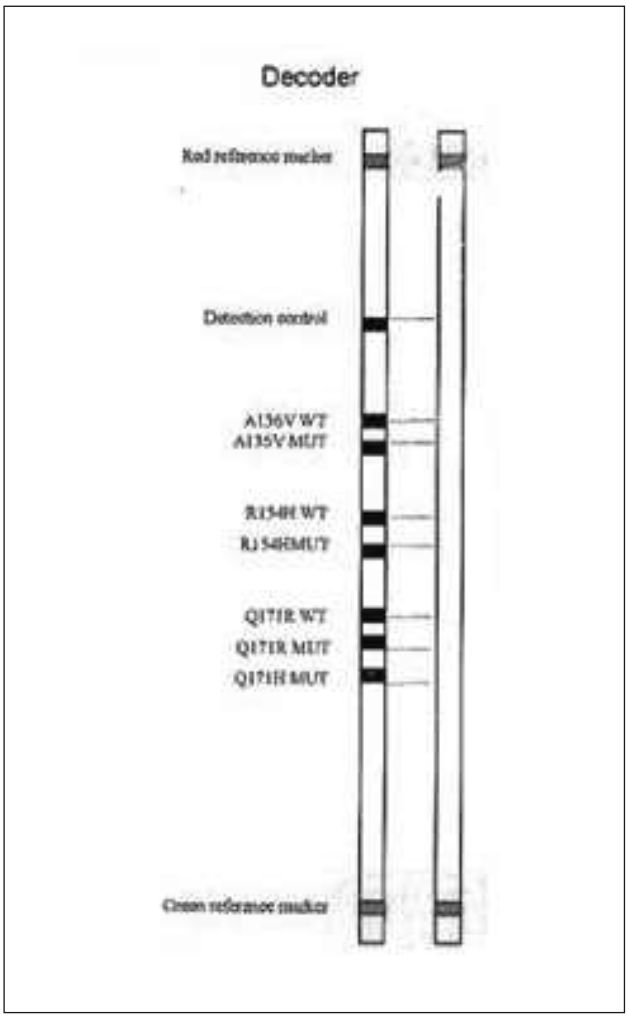

Figure 2. Strip scheme: left strip: decoding strip / right strip: place to put tested strip for evaluation. Red and green reference marker should correspond properly for correct evaluation. "Detection control" on the tested strip stains if test has worked. Other bands (from top to bottom) may appear according to the tested sample: 1st = A, 2nd = V, 3rd = R, 4th = H, 5th = Q, 6th = R and 7th = H.

application, however, a special kit (Qia amp® DNA blood, Qiagen, Venlo, The Netherlands) has to be used for the extraction of the DNA that is not available in the standard kit.

All samples (non-coagulated blood and brain tissue) were also genotyped using DGGE (Bossers *et al.* 1996) and real time polymerase chain reaction (RT-PCR) (Fontaine *et al.*, in preparation) and the results were compared with one another.

The DNA extractions were measured using a spectrophotometer (Gene Quant II RNA/DNA Calculator, Pharmacia Biotech ltd., Cambridge, U.K.). Comparable DNA concentrations of non-coagulated blood and neural tissue were prepared.

RESULTS

Pure extractions of non-coagulated blood revealed an average concentration of 3.28 µg DNA/ml ± 1.41 µg DNA/ml. Pure extractions of brain tissue revealed an average concentration of 14.26 µg DNA/ml ± 5.26 µg DNA/ml. Consequently, the extractions of the brain tissue were diluted 4 times to obtain the same range of DNA concentration as in the extractions of non-coagulated blood.

The positive bands on the strips are identified using the Decoder Table (Figure 2).

One hundred (100) non-coagulated blood samples were tested (Figure 3). The different genotypes found are summarized in Table 1. Ninety-six gave "black and white" results after one test using non-coagulated blood. Three samples had to be tested twice and one

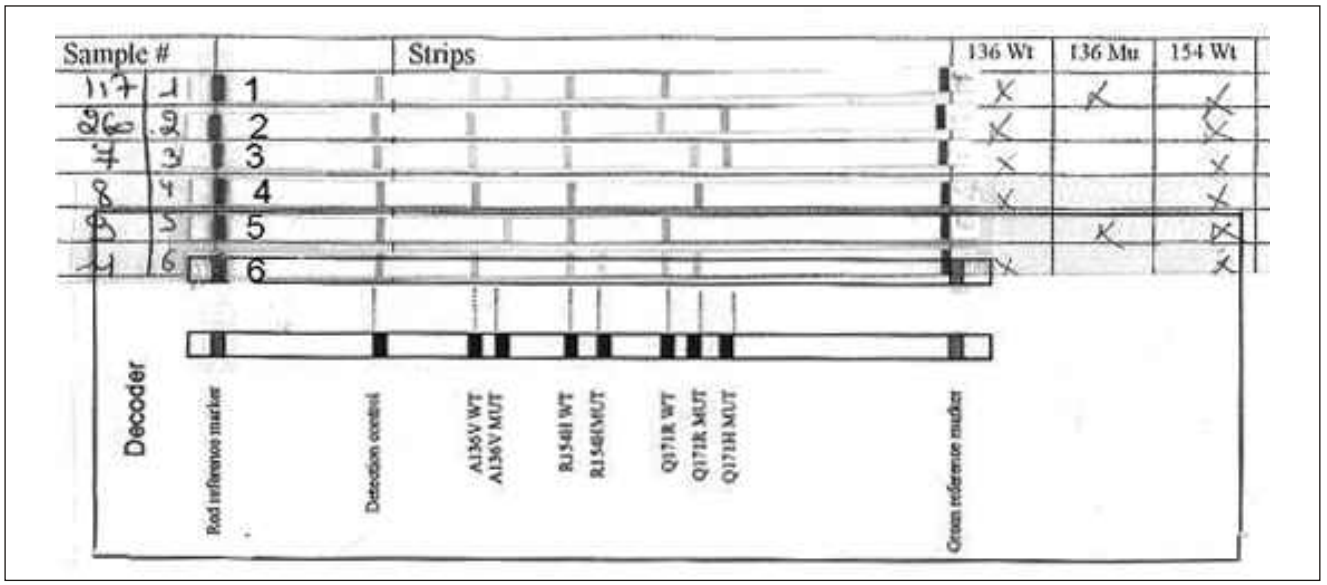

Figure 3. Example of tested strips compared to the decoder. Strip 1: ARQ/VRQ; Strip 2: ARQ/ARH; Strip 3: ARR/ARH; Strip 4: ARR/ARR; Strip 5: VRQ/VRQ; Strip 6: AHQ/ARR.

Table 1. Different genotypes found with the "Ovine PrP Gene Test".

NON-COAGULATED BLOOD SAMPLES	
Genotype	**Number of samples**
AHQ / VRQ	1
ARH / AHQ	1
ARH / ARH	5
ARH / VRQ	4
ARQ / AHQ	5
ARQ / ARH	8
ARQ / ARQ	14
ARQ / VRQ	11
ARR / AHQ	5
ARR / ARH	9
ARR / ARQ	13
ARR / ARR	11
ARR / VRQ	8
VRQ / VRQ	5
Total	**100**
BRAIN TISSUE SAMPLES	
Genotype	**Number of samples**
AHQ / VRQ	2
ARQ / AHQ	1
ARQ / ARH	6
ARQ / ARQ	2
ARQ / VRQ	3
ARR / ARH	5
ARR / ARQ	1
ARR / ARR	3
ARR / VRQ	5
Total	**28**

three times because of the presence of "phantom lines". Finally all samples gave satisfactory results. All results obtained with the "Ovine PrP Gene Test" corresponded with those respectively obtained via DGGE and RT-PCR.

Twenty-eight neural tissue samples were tested (Table 1). The DNA was extracted from spinal cord, cerebellum, cerebrum and post mortal degenerated brain samples. The results of the brain tissue with the present test were identical with the DGGE and RT-PCR results. The "Ovine PrP Gene Test" results of non-coagulated blood and brain tissue of the same sheep were also identical. The signal on the strips obtained using brain tissue was much stronger than the signal on the strips obtained using non-coagulated blood.

DISCUSSION

Allele specific hybridization is generally very sensitive to a number of parameters, especially temperature and DNA quantity and quality. For the Ovine PrP Gene Test, the temperature controlled shaking water bath, which insures the optimal temperature of 45°C and shake-rhythm during the reversed hybridization step, is essential equipment for the test. DNA extracted from brain tissue showed a stronger signal than that of non-coagulated blood (quantity), while older blood samples and postmortal degenerated brain tissue still showed reliable results (quality) (non-published data).

Each of the three techniques used in the present trial have their advantages and disadvantages when compared with one another. This is illustrated by the fact that DGGE may take up to 3 days, while RT-PCR and the Ovine PrP Gene Test require only 4-5 hours to get results. Installation costs are the highest for RT-PCR as compared to DGGE and the Ovine PrP Gene Test. DGGE is limited to 13 samples per run while 20 samples per run could be examined with RT-PCR and 24 samples per run with the Ovine PrP Gene Test. The major advantages of this new "Ovine PrP Gene Test" are its rapidity, its ease of use and manipulation, its need for only a restricted amount of blood (25µl) and its rather low installation and equipment costs as compared to the other available tests. This last argument can be linked mainly to the fact that it uses techniques that are already present in most laboratories.

For all three tests, false test results could be attributed to contamination during manipulation (limited manipulation = reduced risk of contamination) and critical temperature-dependent steps.

In the Ovine PrP Gene Test, false results are expressed as "phantom lines". "Phantom lines" are weak positive signals from the others spots on the strips of a certain codon, which make interpretation difficult. For example: the A136V WT mark on the strip stains very well, while the A136V MUT mark stains very weakly, or vice versa. In this case it is difficult to interpret this result as an AA or AV or VV.

According to the test supplier (NLM), false positive ("phantom lines") and false negative results are mainly attributed to the different steps (e.g. waterbath temperature) being carried out at the wrong temperatures and/or to the used consumables used in the course of the test. In our test evaluation, no real false negatives were detected. However, "phantom lines" were sometimes noted.

The occurrence of "phantom lines" could also be linked to "complex " genotypes as described in Great Britain (Dawson *et al.*, 2003; McKeown *et al.*, 2004). Over one million blood samples have already been genotyped in Great Britain for the National Scrapie Plan (NSP) (Dawson *et al.*, 2003; McKeown *et al.*, 2004). They identified a small proportion of these samples (about 0.1 %) in which it was not possible to report a standard "two allele" genotype. It appeared that these animals carried three or more PrP alleles, such that a result could be reported as ARR/ARR/ARQ or ARR/AHQ/VRQ/VRQ. It is possible that this phenomenon may be a consequence of the relatively high incidence of twinning observed in sheep. Placentary anastomosis (fusion of the placentae of twins, resulting in a common circulatory system during development) (Lillie, 1917) is an attractive theory for explaining these observations and is defined as freemartinism/chimerism (McKeown *et al.*, 2004). This may result in stem cells from one twin being present in the other and vice versa (Szatkowska *et al.,* 1998, Keszka *et al.,* 2001, Verberckmoes *et al* 2002). Although freemartinism/chimerism is likely to occur in only 0.03 % of the entire sheep population (Smith *et al.,* 1998), recent studies indicate that the prevalence of freemartinism in sheep is increasing (Parkinson *et al.,* 2001). The presence of "phantom lines" (4% of the tested cases) in our trial could be attributed to technical glitches, as all 100 tested samples ultimately produced unambiguous results.

In conclusion, we can state that our results showed that the "Ovine PrP Gene Test" appears to be competitive with the present techniques used for PrP genotyping in sheep and that it is suitable for use on blood as well as on brain samples (with an additional kit).

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