DNA Testing Methodology for the Evaluation of Susceptibility to Scrapie in Tsurcana Sheep

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Abstract
Scrapie, first reported in the 18th century, has led to considerable economic losses by affecting the livestock industry. This disease is a transmissible spongiform encephalopathy that mainly affects sheep and goats and is caused by the accumulation of prion protein in an abnormal form (PrPSc) in the nervous system of animals. The identification of genetic polymorphisms in the PRNP gene has allowed the development of effective genetic tests for the detection of susceptibility to Scrapie. In many European countries, these tests are being used to select resistant animals, thereby reducing the risk of spreading the disease in sheep flocks. The method applied in this research to identify susceptible or resistant genotypes to Scrapie in sheep, applies DNA tests made from biological samples such as hair, blood and other tissues. By analysing polymorphisms in the PRNP gene at codons 136, 154 and 171, the animals were classified into five different risk categories. Early genotyping of sheep at the PRNP locus allows rapid selection of genotypes conferring resistance to this disease, thus contributing to improving the genetic resistance of sheep breeds.

Keywords: genetic susceptibility, molecular-assisted selection, PRNP gene, Sanger sequencing, Scrapie control

1. Introduction

This disease mainly affects sheep and goats and is characterized by degeneration of the brain and central nervous system, leading to progressive abnormal behaviour, pruritus (intense itching) and impaired locomotion. The name "Scrapie" comes from the characteristic behaviour of animals scratching obsessively, causing severe skin damage [1]. Scrapie is transmissible and, once established, incurable, which has many countries to seek effective control and eradication solutions.
The history of the disease is long, with the first cases reported in Europe as early as the 18th century, particularly in the UK and other western European countries [2]. Scrapie has remained a major animal health problem with significant economic impact, given the losses caused by the slaughter of animals and quarantine measures imposed on affected farms [3].
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) [4].
Histopathologic examination was the first diagnostic method used and has remained the main means of confirming TSE (Transmissible spongiform encephalitis) in many countries. Depending on the species affected, different areas

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of the central nervous system are examined. The typology of the lesions is identical in all species, varying only in the localization, frequency and intensity of the different histological changes. They are found predominantly in the palencephalon and primarily in the brainstem and medulla. The characteristic lesion is spongiform vacuolization, often symmetrical, of the grey matter neuropil and the presence of vacuoles in the perikaryons [5].

Genetic testing to determine scrapie resistance started to be widely used in the last two decades, with the discovery of the genetic influence on susceptibility to the disease. In the 1990s, it was shown that genetic variations in the Prion Protein Gene (PRNP), which encodes the protein, influence the resistance or susceptibility of sheep to scrapie. In particular, three codons in the PRNP gene - codons 136, 154 and 171 - have been identified as being involved in determining susceptibility to infection [6].

For example, ewes carrying the ARR/ARR genotype are highly resistant to the disease, while VRQ/VRQV genotype are the most susceptible. Genotypes such as ARR/VRQR or ARQ/ARQ confer varying degrees of resistance or susceptibility to the disease [7]. The discovery of these genetic associations has allowed the development of national control programs based on genetic selection of scrapie resistant animals.

In Europe, many countries, including the UK, France and the Netherlands, have implemented national scrapie eradication programs since the 2000s, using selective assisted genetic selection. The UK's National Scrapie Plan (NSP) for example, was one of the most comprehensive programs of its kind, relying on large-scale genotyping of animals and culling of those susceptible animals, particularly those with VRQ genotypes. The main aim of these programs was to increase the frequency of the ARR allele in the sheep population, while animals with the VRQ allele were phased out of breeding schemes [8].

France and the Netherlands adopted similar policies, focusing on genotyping and selection resistant rams to reduce the spread of the disease and protect both public health and the agricultural economy. In many cases, these programs have succeeded in significantly reducing the prevalence of scrapie in flocks contributing to food security and animal welfare.

In Romania, although scrapie has been recognized as a major problem in sheep flocks, genotyping and genetic selection programs to control the disease have been introduced more recently. However, sheep of the Tsurcana breed, which represents one of the most important indigenous breeds, have started to be tested for susceptibility to scrapie, with the aim of obtaining genetic data to enable the development of effective control strategies. Through genetic testing can be determined the ovine genotypes and implement assisted selection programs to increase frequency of resistant genotypes and reduce the risk of future infection.

The importance of eradicating scrapie is underlined by the fact that the disease does not affect only animal welfare, but also the economic security of farmers and the public health, given the potential risk of prion transmission to humans, although this has not been fully proven. The implementation of rigorous genetic testing and selection strategies for resistant animals is a long-term solution to eradicate this disease and to maintain the sustainability of sheep farmers.

In this article we describe in details the steps and laboratory techniques chained in order to generate a methodology to be applied for analysis of susceptibility degree to scrapie. The developed methodology, combined with assisted selection programs, is essential in the efforts to eradicate this disease in small ruminants, with focus on indigenous sheep breeds, such as Tsurcana.

2. Samples collection

Blood samples are collected in sterile vacutainers with anticoagulant. Approximately 3 ml of blood is collected from the jugular vein, ensuring that the collection process is carried out without incidents, such as haemolysis or blood coagulation, which would compromise subsequent genetic analyses. After collection, each vacutainer is gently shaken to ensure proper homogenization between the blood and anticoagulant, thus preventing clot formation and maintaining the sample in a suitable state for subsequent processing [9]. The transport and storage of samples are carried out under controlled conditions (using a refrigerated bag), Shortly after collection the samples are transferred to the laboratory, where the samples are preserved until their analysis by freezing at -20^{o}C. Sample collection, transport and storage is essential for maintaining the stability of the samples until the time of laboratory analyses, preventing their degradation and thus ensuring the accuracy of subsequent results. Sampling is carried out in

accordance with the sanitary-veterinary norm regarding the methodology for sampling, packaging, identification and transport of samples intended for laboratory examinations in the field of animal health and welfare, as well as in the field of genetically modified organisms [10].

3. DNA extraction

For DNA extraction from blood, commercial kits, such as PureLink® Genomic DNA produced by Invitrogen (part of Thermo Fisher Scientific, USA) is used, which provides a standardized and efficient method for obtaining high-quality genomic DNA. This step is essential to prepare the samples for further analyses, such as PCR amplification and sequencing, necessary to determine scrapie susceptible genotypes.

4. DNA purification steps

Preparation of solutions and samples: Before the actual purification process is started, PureLink® Genomic Wash Buffer 1 and PureLink® Genomic Wash Buffer 2 produced by Invitrogen (part of Thermo Fisher Scientific) are prepared by adding absolute ethanol (96%) according to the manufacturer's instructions.

Subsequently, blood samples stored in vacutainers with EDTA anticoagulant are selected [8]. For each sample to be analysed approximately, 200 µL of blood are used, ensuring that each sample was properly treated to minimize the risk of contamination or degradation.

Cell lysis and protein digestion: To release the genomic DNA from the blood cells, are added 20 µL Proteinase K and 20 µL RN-ase to each sample, followed by 200 µL PureLink® Genomic Lysis/Binding Buffer to facilitate dissolution of the cellular components. The samples are then vortexed and incubated at 55°C for 10 minutes to facilitate protein and RNA digestion, thus ensuring efficient cleanup of the genetic material.

DNA binding and purification: After incubation, 200 µL absolute ethanol is added, which facilitated DNA binding to PureLink® Spin colonies by centrifugation at 10,000 x g for 1 min. After this step, the colony-bound DNA is washed in two successive steps with 500 µL PureLink® Genomic Wash Buffer 1 and 500 µL PureLink® Genomic Wash Buffer 2, centrifuging the samples at the specified speed to remove remaining impurities. This process ensures clean DNA, removing contaminants such as residual proteins and RNA. All reagents for these steps are produced by Invitrogen (part of Thermo Fisher Scientific, USA)

Elution of DNA: To obtain purified DNA, 50 µL PureLink® Genomic Elution Buffer is added to PureLink® Spin colonies and incubated at room temperature for 1 minute. Subsequently, by centrifugation at maximum speed for 1 minute, DNA is eluted into sterile 1.5 mL microcentrifuge tubes. Samples are stored at 4°C for short-term analysis or at -20°C for long-term storage.

5. Quality and purity analysis of DNA samples

Once the DNA extraction process is completed, to assess DNA quality and purity, high precision spectrophotometer such as NanoDrop ONE manufactured by Thermo Fisher Scientific, USA, is used to measure DNA concentration and purity (Figure 1.)

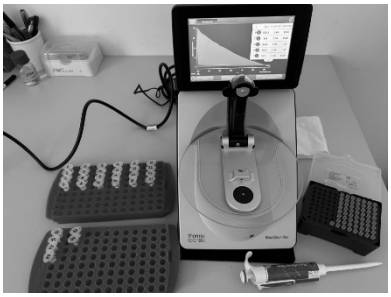

Figure 1. NanoDrop ONE high precision spectrophotometer

Using this method, accurate absorbance values at different wavelengths is obtained to determine the quality and purity of the extracted DNA samples for use in subsequent genetic analysis steps.

In each measurement, the absorbance of the extracted samples is determined at a wavelength of 260 nm, which indicates the DNA concentration. The ratios of the absorbance values measured at 230, 260 and 280 nm (A260/A280 and A260/A230) provide information about the purity of the DNA. Optimal values for the A260/A280 ratio should be between 1.2 and 2.0, indicating adequate purity and absence of protein contaminants. Likewise, an A260/A230 ratio greater than 2.0 indicates the absence of phenolic compounds or other contaminants that could compromise subsequent results.

Following the application of these measurements, it can be ensured that the extracted DNA was of sufficient quality for PCR amplification and sequencing of scrapie susceptible genotypes. This quality control step is essential for reliable and reproducible results in genetic research [11].

6. DNA amplification

To perform amplification of purified DNA, equipments such as thermal cycler produced by BIORAD - USA are used (Figure 2).

To determine the genotypes at codons 136, 154 and 171 of the PRNP gene, the DNA samples extracted and purified in the previous steps are amplified by polymerase chain reaction (PCR) using specific primers flanking the region of interest in the PRNP gene. It is reiterated that the prion protein gene PRNP (Prion Protein Gene) encoding the prion protein PRNP influences the resistance or susceptibility of sheep to scrapie.

PCR reaction steps applied for amplification of the PRNP region

The first step is the preparation of the reaction mixture including extracted and purified DNA, specific primers, nucleotides and Taq polymerase enzyme.

Figure 2. PCR thermal cycler

The DNA amplification program has been structured in several steps, corresponding to initial cycles and main cycles, each with specific times and temperatures.

- Initial denaturation cycle: At the beginning of the amplification, DNA denaturation takes place at 95°C for 3 minutes. This step is necessary to separate DNA double strands, preparing them for primer attachment.
- Master Cycles: After the initial cycle, 40 master cycles are performed, each with three steps:

1. Denaturation: In each main cycle, the DNA is denatured at 95°C for 45 seconds to separate the DNA strands that are renatured.
2. Primer attachment: The next step is the attachment of the specific primers to the DNA strands, performed at 60°C for 45 seconds. This step allows the primers to bind to complementary DNA sequences.
3. Extension of DNA fragments: In the last step of each cycle, DNA fragments are extended (synthesized) using Taq polymerase at 72°C for 1 minute. This step allows the actual synthesis of new DNA fragments by adding nucleotides to the end of the attached primers.

This denaturation, attachment and extension process is repeated for a total of 40 cycles, ensuring amplification of the desired DNA fragments. At the end of the 40 cycles, a final extension step is added at 72°C for 7 minutes to allow for the complete termination of any DNA fragments not fully extended.

7. Agarose gel electrophoresis

After amplification of the region of interest within the PRNP gene, samples of the resulting amplicons are subjected to agarose gel electrophoresis to migrate and separate the DNA molecules amplified in the previous step. After migration of these samples into electrophoresis gels, they are visualized using a lamp emitting light in the ultraviolet (UV) spectrum.

Agarose gel electrophoresis is an essential technique used to separate DNA fragments according to their size. In this step, we went through several work steps, mainly preparing the electrophoresis gel, loading the samples in the electrophoresis tank, migrating them in the electric field and analysing the results [12].

Agarose gel preparation

To migrate the fragments of interest from the PRNP gene, agarose gel is prepared at a concentration of 2%,2 g agarose in a volume of 100 ml TBE 1X migration buffer (Tris-borate 45mM, 1mM EDTA) [12]. This concentration provides an optimal equilibrium for the separation of medium-sized DNA fragments, such as those obtained following PCR amplification of the PRNP gene fragments of interest. The next step is to dissolve the agarose in the TBE liquid by heating the mixture in a microwave oven until the agarose particles are completely dissolved. After the resulting solution is cooled to a temperature of approximately 50°C, a dye or visualization agent is added to allow visualization of the DNA under UV light at the end of the migration process. A GelRed fluorophore is used in this case. There are not toxic UV light visualization agents such as ethidium bromide (EtBr) to reduce

the negative environmental and operator impact of the analysis techniques applied.
The hot liquid (about 50°C) is then poured into the electrophoresis tank fitted with a plastic comb, which creates the wells (spaces) needed to introduce the DNA samples. The liquid after cooling becomes a gel and is allowed to solidify at room temperature for about 20-30 minutes.

Loading samples:
After the gel solidifies, the comb is carefully removed, leaving behind the wells into which the DNA samples will be inserted. In each well, 5 µL of PCR amplification product is added. This is done after an Orange DNA Loading Dye has been added to the DNA sample ,this includes a single dye, bromophenol blue which helps to track migration in daylight and field shift with DNA samples, it also contains EDTA which binds bivalent metal ions and inhibits metal-dependent nucleases and does not mask the DNA during exposure of the gel to UV light, and the presence of glycerol ensures that the DNA in the sample forms a layer at the base of the well .

Migration process:
Once the samples are loaded into the wells, the electrophoresis tank is closed, the terminals from the electric current source are fitted and an electric field is applied. The DNA, being negatively charged, migrates from the negative pole (cathode) to the positive pole (anode) through the porous agarose gel. In the specific case of migration of the amplicons obtained by applying the PCR reaction to amplify the segments of interest within the PRNP gene, a voltage of 70 V is applied and migration lasts 30 minutes until the samples are visibly separated in the electrophoresis gel.

8. PCR product purification and amplification for sequencing
After amplification of DNA fragments by PCR, an essential step is the purification of the PCR products to remove primers, unincorporated nucleotides and other impurities that could interfere with the sequencing step. For this purpose, ExoSAP-IT™ Express PCR Product Cleanup Reagent is used, which provides a fast and efficient method for purification. The reagent is produced by Thermo Fisher Scientific, USA [13].

Purification of PCR reaction products
The steps to purify PCR products are as follows:
1. Addition of ExoSAP-IT™ reagent
For each PCR sample, 5 µL of ExoSAP-IT™ reagent is added to 25 µL of PCR product. This reagent combines two essential enzymes: exonuclease I and shrimp alkaline phosphatase (SAP). Exonuclease I degrades the primers remaining from the PCR reaction, and SAP phosphatase inactivates the unincorporated nucleotides.
2. Incubation for degradation of impurities:
The reaction mixture is incubated for 15 minutes at 37°C to activate the enzymes and allow efficient purification of the PCR product. In this step, the free primers and nucleotides are completely removed from the solution.
3. Enzyme inactivation:
After the purification process, the enzymes were inactivated by incubating the mixture at 80°C for 15 minutes. This ensures that the enzymes will not affect further sequencing.
Purification of the PCR product is crucial to obtain clean and accurate results in the sequencing step by removing components that could compromise further amplification or sequence reading.

Amplification for sequencing
After purification of the PCR products, the purified DNA samples are prepared for the actual sequencing step. This technique allows detailed nucleotide sequence identification in the amplified fragments. In this step, we used the BigDye® Terminator v3.1 Cycle Sequencing Kit by Thermo Fisher, USA [13]. This kit is an efficient and established system in genetic analysis for Sanger-based DNA sequencing.
The working steps for sequencing amplification are as follows:
1. Preparation of the reaction mix for sequencing:
In a total volume of 10 µL, are combined 2 µL of purified DNA, 2 µL of BigDye® Terminator mix, 2 µL of specific primer, and 4 µL of high purity distilled water.
2. Sequencing cycle program:
Samples are loaded into the thermal cycler to perform the amplification cycles. The program consisted of the following steps:
Initial denaturation at 96°C for 1 min.
25 cycles consisting of:
Denaturation at 96°C for 10 seconds.

Primer hybridization at 50°C for 5 seconds.
Extension at 60°C for 4 minutes.
In this step, the specific primers are attached to the DNA templates and polymerase begins synthesizing the DNA sequence, incorporating the fluorescently coloured dideoxy nucleotides (ddNTPs), which will allow the identification of each nucleotide in the sequencing.
3. Post-sequencing purification:
After sequencing amplification, the reaction products are purified using the BigDyeXTerminator™ Purification Kit from Thermo Fisher, USA [13], which is specifically designed for the removal of residual dyes and compounds that may interfere with sequence read-out. This purification is performed according to the manufacturer's protocol to obtain a clear and accurate sequence in the next step of analysis.

9. Sample sequencing

The purified samples are loaded onto an ABI Prism genetic analyser by Thermo Fisher, USA [13] for sequencing. This equipment uses the principle of capillary electrophoresis to separate DNA fragments and detect the fluorescence of incorporated ddNTPs, generating detailed chromatograms for each sequence.
These rigorous purification and sequencing amplification steps allowed us to obtain accurate and reliable genetic data for Scrapie susceptibility assessment [14]. The implementation of this method provides a clear picture of the genetic variation in genes involved in resistance or susceptibility to the disease, thus contributing to the development of more efficient genetic selection strategies in sheep populations.

10. Classification into risk categories

Although this methodology can be applied in small ruminants in general [14], this article is focused on classification of sheep belonging to Tsurcana breed into scrapie risk categories. The application of genetic testing to classify Tsurcana sheep according to scrapie risk enables targeted breeding for resistance.
Risk categories according to genetic resistance or susceptibility to scrapie are the following:
Risk class 1 (R1): genotype ARR/ARR - associated with the highest scrapie resistance.
Risk Class 2 (R2): ARR/AHQ, ARR/ARH, ARR/ARQ genotypes - associated with medium Scrapie resistance.
Risk class 3 (R3): genotypes AHQ/AHQ, AHQ/ARH, AHQ/ARQ, AHQ/ARQ, ARH/ARH, ARH/ARQ, ARQ/ARQ - associated with low scrapie resistance.
Risk class 4 (R4): ARR/VRQ genotype - associated with increased susceptibility to scrapie.
Risk Class 5 (R5): ARQ/VRQ, AHQ/VRQ, ARH/VRQ, VRQ/VRQ genotypes - highly susceptible to the occurrence of the disease.
Incorporating these findings into national breeding programs may reduce scrapie prevalence, safeguard animal health, and strengthen the genetic quality of Tsurcana flocks. Continued research and testing are essential to optimize these selection strategies for sustained livestock improvement.

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