Design and validation of a high-throughput assay to detect codon 146 polymorphisms in the caprine prion protein gene

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A B S T R A C T

Scrapie is a neurodegenerative prion disease that affects sheep and goats, leading invariably to the death of affected animals. As in other prion diseases, it is linked with accumulation of PrPSc, the pathological isoform of the cellular prion protein (PrPc), mainly in the central nervous system [1,2]. In sheep, but not in goats, the strong association of scrapie susceptibility with certain single nucleotide polymorphisms (SNPs) in the PrP gene constitutes the basis for selective breeding strategies directed toward controlling the disease in ovine populations.

Similar to the sheep PrP gene, the caprine PrP gene is highly polymorphic. To date, 24 polymorphic positions resulting in nonsynonymous SNPs of the caprine PrP gene have been reported in different breeds [3–11]. In addition, Goldmann and coworkers [4] described a smaller version of the PrP gene that encodes a protein variant comprising only three octapeptide repeats at the N terminus. Several authors have studied the association of polymorphisms at the PrP gene with scrapie susceptibility in goats. Case studies in Italy and France involving a variety of goat breeds have demonstrated a protective role for allele K222 [9,12,13] against classical scrapie. Similarly, the substitution of glutamine for arginine at codon 211 has been associated with an increase in resistance to classical scrapie in French Alpine and Saanen goats [13]. Allele H154 has been associated with limited resistance to naturally occurring classical goat scrapie in case studies from Greece, France, Italy, and Cyprus [5]. The presence of allele M142 has been linked with increased disease incubation time in goats experimentally challenged with classical scrapie or bovine spongiform encephalopathy (BSE) [3] and with increased scrapie resistance in a French field study [13]. A recent report examining PrP genotypes in Cypriot goats of the Damascus and mixed Damascus/indigenous breeds revealed a robust association between polymorphisms at codon 146 of caprine PrP and scrapie susceptibility/resistance [10]. Specifically, whereas the codon 146 aspartic acid (D) and serine (S) substitutions appear to provide protection against scrapie in Cypriot goats, the presence of the wild-type N146 allele in homozygosity is strongly linked with susceptibility [10].

The aim of the current study was to develop a high-throughput assay, based on homogeneous MassExtend (hME) technology [14] coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS), for genotyping codon 146 of the caprine PrP gene. Primer extension methods such as hME are flexible and robust, and they offer the possibility to interrogate several positions in a single reaction tube (multiplexing) [15]. MALDI–TOF MS offers fast speed of analysis, high accuracy, and the ability to genotype multiple SNPs in a single experiment

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2 Abbreviations used: PrP, prion protein; SNP, single nucleotide polymorphism; BSE, bovine spongiform encephalopathy; hME, homogeneous MassExtend; MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PCR, polymerase chain reaction; SAP, shrimp alkaline phosphatase; ORF, open reading frame; TSE, transmissible spongiform encephalopathy.
[15]. Primer extension coupled with MALDI–TOF MS has been used successfully for large-scale SNP genotyping projects, including the National Scrapie Plan for Great Britain that oversees SNP genotyping of sheep PrP (codons 136, 154, and 171).

Materials and methods

Sample information and DNA extraction

In this study, a total of 731 goats from four different herds of the island of Cyprus were sampled and analyzed. Herds A and B were composed of animals of Damascus breed, herd C was composed of animals of Machaeras breed, and herd D was composed of crossbred animals. Herd A was the only scrapie-affected herd. However, only healthy animals were tested in this study.

Goat blood samples were collected directly onto FTA blood cards (cat. no. WB12 0305, FTA Classic Card, Whatman) following the manufacturer’s instructions. Genomic DNA was extracted with the Chemagic DNA Blood Spot Kit (cat. no. 1030, Chemagen, Bae-sweiler, Germany) using the automated DNA separation module Chemagic module I with a 96-well metal rod head (Chemagen).

PCR amplification

To prepare templates for the hME reactions, a 254-bp segment of the goat PrP gene encompassing codon 146 was amplified with the primers GEN_ALT_1_F (5′-TG CTT TGG ATG GGC CAA GGT AGT GC-3′) and GEN_ALT_1_R (5′-GAC GTT GGA TGC ACC ACT CGC TCC ATT ATC T-3′). These primers contain 5′ tags, described in the GenBank entryAY326330 for primers OARPRPDS1:399U17 and OARPRPDS1:616L19, to increase the primer masses so that they are outside the resolving range of the MALDI–TOF mass spectrometer. Amplification was performed in 8-μl reactions containing 1× HotStar Buffer, 0.2 mM of each dNTP, 240 nM of each primer, 2.5 mM MgCl2 (final concentration), and 2 μl of genomic DNA preparation (4–12 ng). All polymerase chain reactions (PCRs), as well as subsequent shrimp alkaline phosphatase (SAP) and hME reactions, were set up in 384-well plates using an epMotion 5070 li-quid handling robot (Eppendorf, Hamburg, Germany). Microplates were sealed with Peel-it-Lite foil sheets (Eppendorf) using an Eppendorf Heat Sealer, and thermal cycling was performed in an Eppen-dorf Mastercycler ep384.

Thermal cycling conditions were as follows: initial denaturation for 10 min at 95 °C, followed by 55 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s, followed by a 3-min final extension at 72 °C. Unincorporated dNTPs in the PCR reactions were dephosphorylated by treatment with SAP. Aliquots (2 μl) of SAP mix, containing 1.32 μl of water, 0.2 μl of hME reaction buffer (cat. no. 10055, Sequenom), and 0.48 μl of SAP (1 U/μl, cat. no. 10002, Sequenom), were added to each well, and the reaction plate was incubated for 20 min at 37 °C. SAP enzyme was subsequently heat inactivated at 85 °C for 5 min.

hME reactions

Duplex hME reactions were carried out with the addition of 2 μl of hME master mix to the dNTP-treated PCR products. The composition of the hME master mix was as follows: 0.2 μl of hME reaction buffer, 0.6 μl of each extension primer, 0.024 μl of the appropriate termination mix, and 0.0236 μl of ThermoSequenase (32 U/μl, cat. no. 10052, Sequenom). The termination mixes used in this study were as follows: GC (ddGTP, ddCTP, ddATP, and dTTP, cat. no. 10042, Sequenom), AC (ddATP, ddGTP, ddCTP, and dTTP, cat. no. 10039, Sequenom), and AT (ddATP, ddGTP, ddCTP, and dTTP, cat. no. 10041, Sequenom). Sequences and masses of the primers and the predicted products of their extension with the three termination mixes are described in Table 1. Thermal cycling conditions for the hME reactions were as follows: 94 °C for 2 min followed by 99 cycles of 5 s at 94 °C, 5 s at 52 °C, and 5 s at 72 °C. Prior to MALDI–TOF analysis, reaction mixtures were diluted by the addition of 13 μl of water and then were desalted during a 20-min incubation with 6 mg of MassArray Clean Resin (cat. no. 08040, Sequenom) on a rotating shaker at room temperature. Nanoliter volumes of the desalted reaction products were dispensed onto 384-well SpectroChips (cat. no. 00637, Sequenom) using a RoboDesign Nanodispenser (RoboDesign, Carlsbad, CA, USA), and the SpectroChips were subsequently analyzed using the Bruker Biflex III mass spectrometer (Bruker Dalton-ics, Bremen, Germany). The acquired spectra were analyzed using MassArrayTyper software (version 3.1.4.0, Sequenom).

DNA sequencing

The entire PrP open reading frame (ORF) was amplified from genomic DNA with the primers Pr3_F (5′-TGG GCA TAT GCT GCT GAC AC-3′) and Pr3_R (5′-AAA CAG GAA GGT TGC CCC TA-3′) in 15-μl reactions with the same composition as described in the “PCR Amplification” section above except that the concentration of each primer was 1 μM and 3 μl of genomic DNA preparation was used per reaction. Thermal cycling conditions were as described previously [9]. DNA sequencing reactions were performed with a BigDye Terminator (version 3.1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using primers P8 and P9, as described previously [9].

Results

hME assay design

In hME assays, interrogation primers are extended in the presence of appropriate ddNTPs and dNTPs (termination mix) to give al-

<table>
<thead>
<tr>
<th>Termination mix</th>
<th>Primer sequence</th>
<th>Mass (Da)</th>
<th>Encoded amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>GCCCTTATGATTTTGC</td>
<td>5760.7</td>
<td>A</td>
</tr>
<tr>
<td>G_146_F</td>
<td>GCCCTTATGATTTTGC</td>
<td>6074.0</td>
<td>D</td>
</tr>
<tr>
<td>G_146_R</td>
<td>GCCCTTATGATTTTGC</td>
<td>7004.6</td>
<td>N</td>
</tr>
<tr>
<td>AT</td>
<td>GCCCTTATGATTTTGC</td>
<td>6387.2</td>
<td>S</td>
</tr>
<tr>
<td>G_146_F</td>
<td>GATGATACGCTTATGCTATCA</td>
<td>7047.6</td>
<td>S</td>
</tr>
<tr>
<td>G_146_R</td>
<td>GATGATACGCTTATGCTATCA</td>
<td>7625.0</td>
<td>D</td>
</tr>
<tr>
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<td>7969.2</td>
<td>N</td>
</tr>
<tr>
<td>G_146_R</td>
<td>GATGATACGCTTATGCTATCAG</td>
<td>7320.8</td>
<td>S</td>
</tr>
<tr>
<td>AC</td>
<td>GCCCTTATGATTTTGC</td>
<td>5760.7</td>
<td>A</td>
</tr>
<tr>
<td>G_146_F</td>
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<tr>
<td>G_146_R</td>
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<td>7320.8</td>
<td>S</td>
</tr>
</tbody>
</table>

4 GC: ddGTP, ddCTP, ddATP, and dTTP; AC: ddATP, ddCTP, ddGTP, and dTTP; AT: ddATP, ddGTP, ddCTP, and dTTP.

Nucleotides added to the primers during the extension reaction are shown in bold.
The four nucleotides corresponding to the full N_146 extension peak indicates that a single nucleotide has been added instead of incomplete extension of the forward primer. The mass of this peak is likely a pausing peak attributable to the reverse assay was confirmed by DNA sequencing. ND in the forward assay. The accuracy of the genotype calls obtained for the forward and reverse assays. Up to 70% of the samples that were called NN in the reverse assay were called NS. The genotype distribution in these two groups is not statistically different (P = 0.15385). In both cases, tested animals belonged to the Damascus breed and came from herds that had been stricken by scrapie. Despite the fact that the populations tested were quite different (278 goats from the one herd [A] in this study vs. 54 animals from 12 different herds in the previous study), the genotype distributions are very similar in both cases and are significantly different from the genotype distribution of the control herds B (Damascus animals unaffected by scrapie). These results suggest that, in the Damascus breed, scrapie is selecting against NN animals.

**Discussion**

We have developed a highly accurate and reproducible assay for genotyping codon 146 variants of the caprine PrP gene using Sequenom’s MassARRAY system with hME technology. The MassARRAY platform is exceedingly user-friendly and was designed to require little operator input. The entire analytical process (DNA amplification, SAP treatment, primer extension, and desalting of the extension products) takes place in a single well (homogeneous reactions), thereby avoiding volume losses and cross-contamination events. The estimated price for the codon 146 genotyping assay in our laboratory is 1.60 euros or 2.25 US dollars, per sample. This price calculation is based on prices in Greece at the time of this writing and includes reagents, plasticware, and SpectroChips required for the PCR amplification and hME assay preparation and analysis. Genomic DNA extraction cost is not included. This high-throughput assay is suitable for genotyping large numbers of samples, as required for transmissible spongiform encephalopathy (TSE) surveillance projects. It allows efficient
screening of large goat populations for identification of individuals carrying the codon 146D and 146S alleles. In addition, it could easily be incorporated into a multiplex assay to simultaneously screen for polymorphisms at other codons, as is routinely done in sheep genotyping programs that check for polymorphisms at codons 136, 154, and 171 of PrP.

Currently, genetic studies of goat scrapie are not as advanced as those for sheep. To date, polymorphisms at codons 142, 143, 146,
154, 211, and 222 have been linked with prolonged scrapie incubation time or scrapie resistance/susceptibility in different breeds and geographical regions [3,5,9,10,12,13]. It is likely that the number of “meaningful” polymorphisms will increase in the future as genetic characterization of the goat PrP gene progresses. An ideal large-scale animal screening assay for scrapie resistance would include all PrP SNPs linked with scrapie predisposition according to breed and/or geographical regions. As mentioned above, hME assays are generally compatible with multiplexing, which allows interrogation of several positions of the gene in a single well, thereby effectively multiplying the information obtained without increasing the cost substantially. When used in this way, hME assays can provide a very cost-effective method for SNP analysis. It must be noted, however, that there could be one potential drawback in the practical application of multiplex hME, or any other multiplex primer extension-based technique, to scrapie susceptibility studies. This stems from the fact that all of the implicated SNPs are located in the PrP gene, thereby allowing the possibility that relevant SNPs might be located in neighboring positions. In such cases of close SNP proximities, the extension primers designed to interrogate neighboring positions would overlap and likely interfere with each other. Even in such circumstances, however, all SNPs of interest could be determined by separating incompatible extension primers into separate reactions.

To summarize, we have designed an efficient and cost-effective high-throughput assay to detect genetic polymorphisms at codon 146 of the caprine PrP gene, a position known to be polymorphic in the goat populations of Cyprus, China, Japan, and the United States [7,8,10,16]. Furthermore, we applied this assay to the genotyping of codon 146 in 731 goats originating from Cyprus, a geographical area where codon 146 polymorphisms have been linked with classical scrapie predisposition, with substitutions of aspartic acid and serine appearing to be associated with significant disease resistance. Analysis of these goat samples showed that Damascus breed exhibits a remarkable variability at codon 146 of PrP and suggests that scrapie exposure might be affecting the genotype distribution in this breed. Further genetic analysis of healthy and scrapie-affected flocks is required to confirm this possibility.

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References