Article

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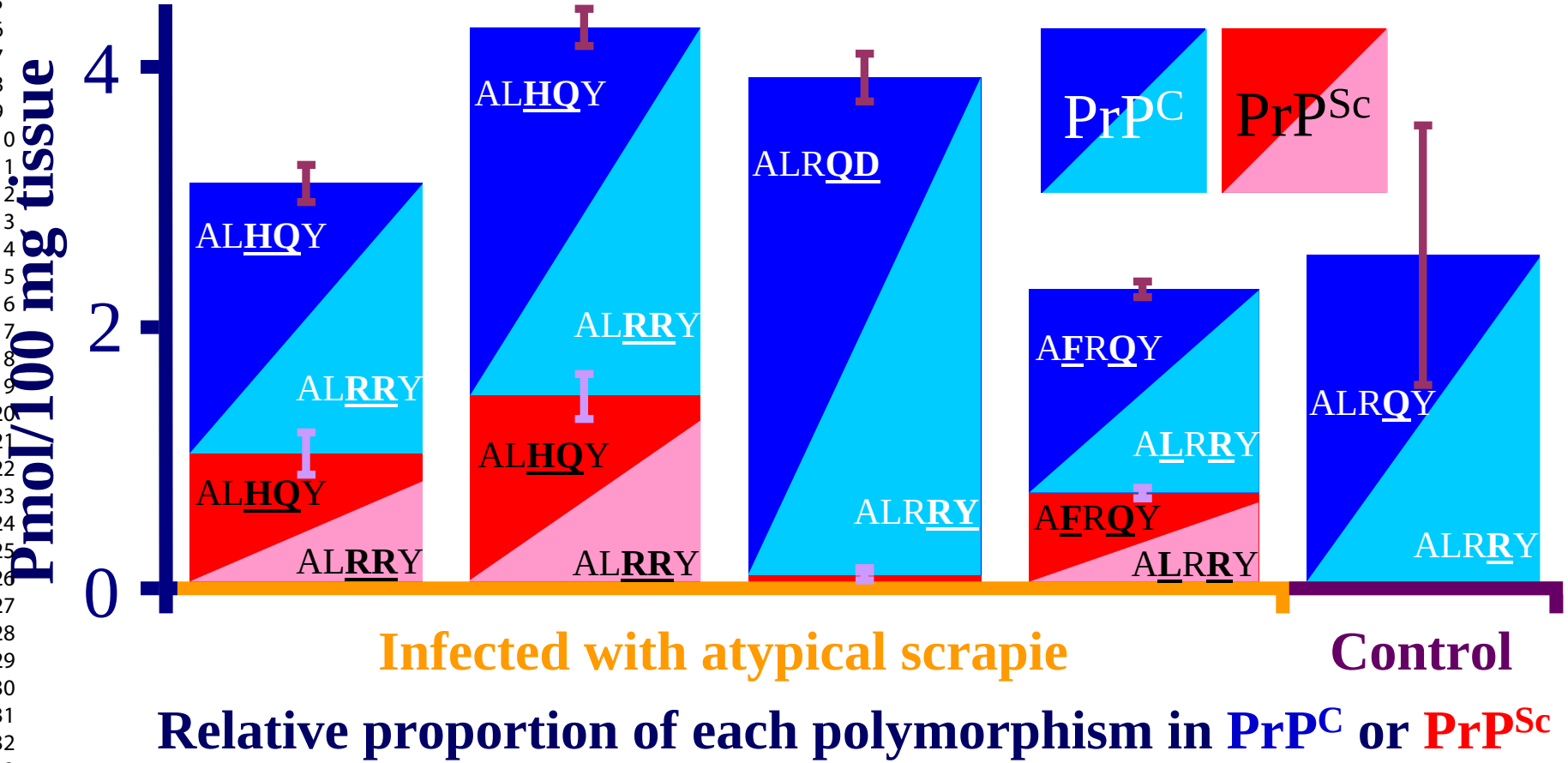

Relative proportion of each polymorphism in PrP^{C} or PrP^{Sc}

Title: Determining the relative susceptibility of four PrP genotypes to atypical scrapie.

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Abstract

Atypical scrapie is a sheep prion (PrP^{Sc}) disease whose epidemiology is consistent with a sporadic origin and is associated with specific polymorphisms of the normal cellular prion protein (PrP^{C}). To determine the relative amounts of PrP polymorphisms present in scrapie, total PrP was digested with chymotrypsin to generate characteristic peptides spanning relevant polymorphisms at positions 136, 141, 154, 171 and 172 of sheep PrP^{C}. A multiple reaction monitoring method (MRM), employing ^{15}N-labeled internal standards, was used to detect and quantify these polymorphisms present in both the PrP^{Sc} and PrP^{C} from heterozygous (ALRRY and ALHQY or ALRQD or AFRQY) atypical scrapie-infected or uninfected control sheep. Both polymorphisms of the full length and truncated (C1) natively expressed PrP^{C} are produced in equal amounts. The overall amount of PrP^{C} present in the infected or uninfected animals was similar. PrP^{Sc} isolated from heterozygotes was composed of significant amounts of both PrP polymorphisms, including the ALRRY polymorphism which is highly resistant to classical scrapie. Thus, an atypical scrapie infection does not result from an overexpression of sheep PrP^{C}. The replication of all atypical scrapie prions occurs at comparable rates, despite polymorphisms at positions 141, 154, 171, or 172.

Introduction

Prions (PrP^{Sc}) are infectious proteins that induce a native cellular prion protein (PrP^{C}) to refold and adopt the PrP^{Sc} conformation.[1] Scrapie is the prototypical prion disease and has been known since the 18th century.[2] The prototypical or classical scrapie was shown to be transmissible among sheep with vulnerable genotypes.[3] Near the end of the 20th century, a new form of scrapie was discovered.[4] This new form is referred to as atypical or Nor98 scrapie to distinguish it from the classical form of scrapie.[5] Atypical scrapie has been experimentally transmitted to sheep and transgenic mice.[6, 7] Classical scrapie is spread among proximate animals and from contact with contaminated environments.[3] Transmission of classical scrapie is strongly influenced by polymorphisms at positions 136, 154, or 171 in the native prion protein (PrP^{C}), by facilitating or impeding to a varying extent the refolding/propagation process.[8-13] Sheep that are homozygous for arginine (R) at position 171 are almost completely resistant to classical scrapie and those animals heterozygous for R at 171 are highly resistant to classical scrapie. In contrast, atypical scrapie is found in sheep with genotypes that are resistant to classical scrapie.[4, 14, 15]

As more extensive transmissible spongiform encephalopathy (TSE) screening programs were implemented, analysis of the sheep samples revealed that the incidence of atypical scrapie is comparatively rare and constant in the 21st century.[5, 16] The etiology of atypical scrapie is consistent with a spontaneous origin.[5, 15, 17-20] It typically afflicts a single animal in a flock and does not appear to be efficiently spread among other sheep in that flock. Atypical scrapie is even found in countries and flocks that are free of classical

scrapie.[21-23] Classical scrapie is spread throughout the lymphatic system of an infected animal, while the distribution of atypical scrapie is largely restricted to the brain of an infected animal, although it can be found in peripheral tissues.[24] A number of samples of atypical scrapie with varied genotypes were transmitted to transgenic mice via intracranial (*ic*) inoculation.[6, 25-28] Even though the samples had very different genotypes, they reproduced the atypical phenotype, had a 100% attack rate, and had comparable incubation periods.[6] Atypical scrapie has been transmitted to sheep by *ic* inoculation and oral inoculation.[7, 29-32] Even though atypical scrapie is orally transmissible, the epidemiological evidence is consistent with a sporadic or spontaneous etiology, suggesting the practical transmission rate is low.

PrP^{C} and PrP^{Sc} are isosequential and only differ in their respective conformations. PrP^{C} is a monomer with no resistance to proteinase K (PK) digestion, while PrP^{Sc} is a multimer with significant resistance to PK digestion. PrP^{Sc} may form different conformations or strains of scrapie, including classical and atypical, each with its own distinct incubation period and pathological phenotype. Each strain is thought to be a distinct conformation. When classical scrapie is digested with PK and then analyzed by Western blot, a characteristic set of N-terminal truncated bands are observed. The bands migrate between 27 and 30 kDa and are referred to as PrP27-30.[33] PrP27-30 includes amino acids at positions 136, 154, and 171. Digestion of atypical scrapie with PK yields a characteristic band of approximately 12 kDa that, unlike classical scrapie, is both N-terminally and C-terminally truncated.[4, 34-36] This band contains amino acids from approximately position 90 to 150.[34-36] It is analogous to that observed in Gerstmann-Sträussler-Scheinker (GSS) disease, an inherited human prion disease.[37]

Atypical scrapie is more commonly associated with sheep expressing polymorphisms that are not associated with classical scrapie.[4, 5, 14, 15, 26, 34, 38-44] The transmission of classical scrapie is strongly related to polymorphisms at positions 136 and 171 and to a lesser extent with polymorphisms at position 154. Sheep expressing the $A_{136}Q_{171}$ polymorphism are highly susceptible to classical scrapie, while those expressing the $A_{136}R_{171}$ polymorphism are resistant to the majority of classical scrapie strains save one.[45] Atypical scrapie has been found in animals expressing either the $A_{136}Q_{171}$ or $A_{136}R_{171}$ polymorphism. However, polymorphisms at positions 141 (F) and 154 (H) are disproportionately associated with atypical scrapie, while the 154 (H) polymorphism is associated with resistance to classical scrapie.[4, 5, 14, 15, 41, 43] Atypical scrapie is found infrequently in a flock and usually as a single case, even though other flock mates share the same genotype. This suggests that it is not a heritable disease.

In transgenic mice experimentally infected with atypical scrapie, PrP expression levels are related to incubation periods of the subsequent disease.[26] Those transgenic animals expressing higher levels of PrP^C succumb to prion disease earlier than those lines expressing lower levels of PrP^C.[25, 26] Transgenic animals engineered to express PrP^C associated with heritable prion disease succumb to the heritable disease only when the expression levels of PrP^C are sufficiently high (3x normal expression levels).[46] Determining the amount of PrP^C in brain samples of sheep infected with atypical scrapie would provide insight into the role of PrP^C expression levels in the development of these spontaneous diseases.

We have developed a method of quantifying the polymorphisms in sheep PrP.[47] Unlike other methods it is not dependent upon the availability of antibodies and will work

with the typical polymorphisms at positions 136 (A and V), 154 (R and H), and 171 (H, K, Q, and R).[48, 49] It employs chymotrypsin,[47] instead of trypsin,[50, 51] to cleave the denatured prion protein into peptides that are suitable for a multiple reaction monitoring (MRM)-based analysis. This approach yields peptides that are not covalently bound to the post-translational modifications of PrP^{C}, the glycosylphosphatidylinositol (GPI) anchor or asparagine (N) bound multimeric sugar residues. This method is a convenient means of quantifying the polymorphisms in sheep PrP.

We have expanded a mass spectrometry-based MRM method of detecting and quantitating the PrP polymorphisms present in a sample of PrP^{Sc} from a heterozygous prion infected sheep. We have applied this method to a set of heterozygous sheep naturally infected with atypical scrapie and report the results here.

Experimental Procedures (Materials and Methods)

Chemicals. The sheep peptides (GQPHGGGW, M_{132}LGSAMSRP**F**$_{141}$ and Y_{166}RPVDQ**D**SNQNNF$_{178}$) were obtained from Elim Biopharmaceuticals (Hayward, CA). Throughout the manuscript, the relevant polymorphisms (variations in the amino acids at positions 136, 141, 154, 171, or 172) are bolded and underlined. Mass spectrometry was used to confirm the chemical composition of all of these synthetic peptides. The peptides were at least 95% pure based on LC/UV-based analysis.

Appropriate ^{15}N-labeled recombinant PrP proteins were digested with chymotrypsin to yield the required ^{15}N-labeled internal standards. The sequence of each PrP protein was determined by sequencing the clone used to express it and further

confirmed by mass spectrometry. The incorporation of ^{15}N label into the PrP samples was estimated to be 99.7%.

Preparation of the sheep recombinant PrP polymorphisms. Site directed mutagenesis was used to prepare genes that would express sheep PrP polymorphisms at positions 141 (F) and 172 (D) using standard molecular biology techniques (Supporting Information).

Preparation of the ^{15}N-labeled internal standards. The two clones expressing the desired sheep PrP genes were each cloned into BL21 cells. Each clone was separately grown in minimal medium supplemented with $^{15}NH_4Cl$ and induced to express its respective PrP polymorphism (Supporting Information).

Genotypes of the sheep samples. The *PRNP* gene from each sheep was sequenced to determine the amino acid sequence of the PrP^C in each sample (Figure S-1 and Table S-2, Supporting Information).

Quantitative Mass Spectrometry: Nanospray LCMSMS. An Applied Biosystems (ABI/MDS Sciex, Toronto, ON) model 4000 Q-Trap instrument with a nanospray source and a nanoflow LC was used to analyze the samples (Supporting Information).

Safety considerations

Hazardous material, such as acetonitrile, was manipulated in a dedicated chemical safety hood. Scrapie is infectious; therefore all manipulations of scrapie-containing samples were performed in a dedicated biosafety level 2 (BSL2) laboratory (Supporting Information).

Results and Discussion

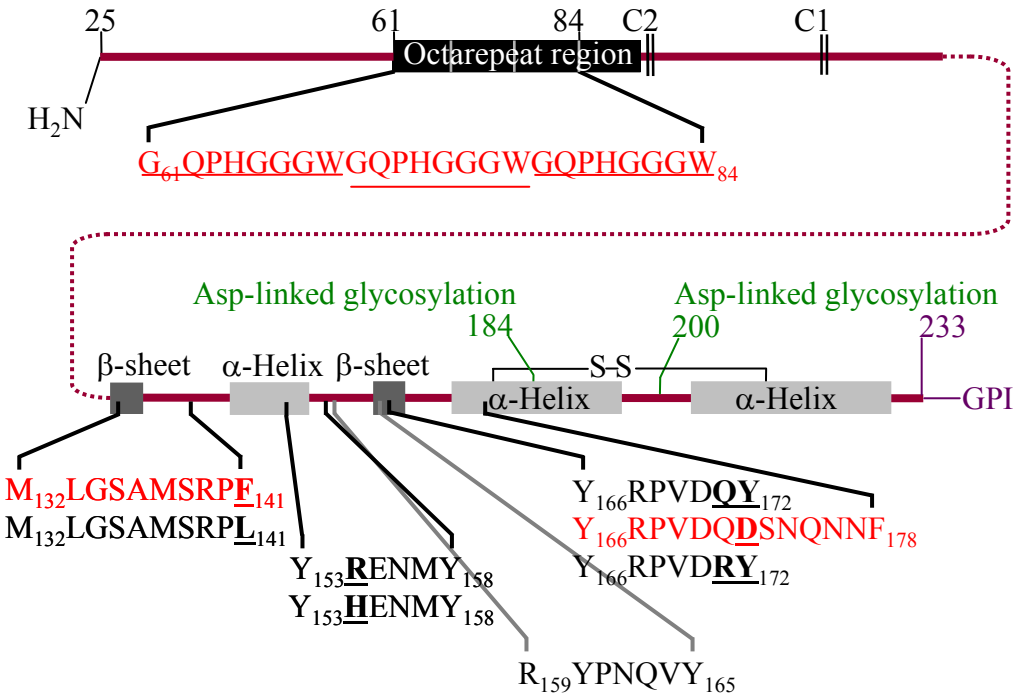

Figure 1. Cartoon of sheep PrP. Important primary and secondary structures, octarepeat region (61-93), β-sheets (131-135 and 164-168), and α-helices (146-156, 174-195 and 204-229) are indicated.[52] Positions of covalent post-translational modifications, a single disulfide bond (S-S; cysteine 182 and 217), two asparagine (Asp)-linked glycosylation sites (asparagine 184 and 200), and a C-terminal glycosylphosphatidylinositol (GPI) anchor are indicated. Relevant chymotryptic peptides in the octarepeat region, and those spanning the 136, 141, 154, 171, and 172 polymorphisms, and the analyte peptide ($R_{159}YPNQVY_{165}$), are indicated by location and sequence. C1 and C2 cleavage sites are indicated by the parallel lines. Peptides shown in red are described in this work. Those in black are described in previous work.[47]

Identification of characteristic analyte peptides. Our previous work identified six chymotryptic peptides (M_{132}LGS**X**MSRPL$_{141}$ [**X** = A or V], Y_{153}**X**ENMY_{158} [**X** = R or H], or Y_{166}RPVD**X**Y_{172} X = Q or R) that can be used to quantify polymorphisms at positions 136, 154 and 171 of sheep PrP.[47] Polymorphisms at position 141 (L or F) of sheep PrP are statistically associated with atypical scrapie, while the rare polymorphism at position 172 (D) is not.[53] Sheep PrP (Figure 1 and Figure S-1, Supporting Information) was digested with chymotrypsin (*in silico* using the ExPASy portal; http://www.expasy.org/tools/). *In silico* digestion predicted several peptides that would be suitable for quantitating polymorphisms at positions 141 and 172: M_{132}LGSAMSRP**X**$_{141}$ (**X** = L; previous work or F; this work), Y_{166}RPVDQ**D**SNQNNF_{178} (with the D polymorphism; this work) and the previously described peptides with the 172 Y polymorphism, Y_{166}RPVD**X**Y_{172} (**X** = Q or R).[47] Each was suitable for a multiple reaction monitoring (MRM)-based analysis. Based on this analysis and previous work, chymotrypsin would yield a set of peptides suitable for quantifying relevant sheep PrP polymorphisms at positions 136, 141, 154, 171, and 172.

In this manuscript we adopt a shorthand convention to distinguish among the sheep PrP polymorphisms. The entire sheep PrP protein sequence (25-233) is summarized by listing five single letter amino acid codes and their subscripted positions in the protein. For example, the sheep PrP indicated by the shorthand $A_{136}L_{141}R_{154}Q_{171}Y_{172}$ has an alanine (A) at position 136, leucine (L) at position 141, arginine (R) at position 154, glutamine (Q) at position 171, and a tyrosine (Y) at position 172 (Figure S-1, Supporting Information). Those amino acids and their positions not listed are common to all of the sheep PrP variants used in this work.

Recombinant sheep PrP ($A_{136}L_{141}R_{154}Q_{171}\underline{\mathbf{Y}}_{172}$), was digested with chymotrypsin to experimentally verify the *in silico* predictions. Qualitative analysis of the chymotryptic digest of $A_{136}L_{141}R_{154}Q_{171}Y_{172}$ revealed the presence of the predicted peptide, M_{132}LGSAMSRP$\underline{\mathbf{L}}_{141}$ that could be used to quantitate polymorphisms at position 141.

A second sheep rPrP ($A_{136}L_{141}R_{154}Q_{171}\underline{\mathbf{D}}_{172}$), was digested with chymotrypsin to verify the *in silico* predictions. The peptide, M_{132}LGSAMSRP$\underline{\mathbf{L}}_{141}$, observed from the first digestion (*vide supra*), was also observed in this digestion. The peptide Y_{166}RPVDQ$\underline{\mathbf{D}}$SNQNNF$_{178}$ was observed in this digestion, but not in the other digest. This distinct peptide could be used to quantify the D_{172} polymorphism. The molar signal intensity of Y_{166}RPVDQ$\underline{\mathbf{D}}$SNQNNF$_{178}$ is much less than that of the analogs with the Y_{172} polymorphism (Y_{166}RPVD$\underline{\mathbf{X}}Y_{172}$ ($\underline{\mathbf{X}}$ = Q or R), which influences quantitation of samples with low amounts of PrP^{Sc} (*vide infra*).

Peptide Y_{166}RPVDQ$\underline{\mathbf{D}}$SNQNNF$_{178}$ was unaffected by overnight digestion (ttest; $p > 0.05$). The same is true for the peptide M_{132}LGSAMSRP$\underline{\mathbf{F}}_{141}$ and as was previously shown with its analog, M_{132}LGSAMSRP$\underline{\mathbf{L}}_{141}$.[47] These results indicate that overnight digestion with chymotrypsin does not significantly degrade these chymotryptic peptides.

Preparing the sheep rPrP polymorphisms. Two clones, $A_{136}\underline{\mathbf{F}}_{141}R_{154}Q_{171}Y_{172}$ and $A_{136}L_{141}R_{154}Q_{171}\underline{\mathbf{D}}_{172}$, were prepared. Each was expressed from a gene encoding the mature (25-233) PrP, which lacks the N-terminal and C-terminal cleaved signal sequences. Site directed mutagenesis of the gene (in a pET-11a vector) was used to generate clones expressing the two sheep PrP polymorphisms at positions 141 or 172 not

done in our previous work (Supporting Information). The mutated genes were verified by sequencing. The two clones were used to prepare the required sheep rPrP.

The plasmids were cloned into the *E. coli* BL21 cell line. These cells were grown in minimal medium to produce the required rPrP, or grown in minimal medium with $^{15}NH_4Cl$ as the sole nitrogen source to produce stable-isotope (^{15}N) labeled protein for use as an internal standard. The resulting PrP proteins were separately isolated as inclusion bodies and then purified by immobilized metal (Cu^{2+}) chromatography (IMAC). Proteins were very pure (> 90%) and the ^{15}N-labeled proteins had a very high isotopic abundance (> 99.7%). The natural abundance (^{14}N) proteins were used to verify the predicted *in silico* results.

Verifying the other sheep rPrP polymorphisms behave as predicted. Three sheep rPrPs ($A_{136}L_{141}R_{154}Q_{171}Y_{172}$, $A_{136}F_{141}R_{154}Q_{171}Y_{172}$, and $A_{136}L_{141}R_{154}Q_{171}D_{172}$) were separately digested with chymotrypsin. Qualitative mass spectrometric analysis of the three proteins showed the presence of the predicted three peptides, $M_{132}LGSAMSRP\underline{\mathbf{X}}_{141}$, (**X** = L or F), and $Y_{166}RPVDQ\underline{\mathbf{D}}SNQNNF_{178}$ from this and other previously described work.[47] These results confirmed that chymotryptic digestion will yield a set of peptides suitable for quantifying the sheep polymorphisms at positions 141 (L or F) and 172 (D). The previously described peptides ($M_{132}LGS\underline{\mathbf{X}}MSRPL_{141}$ [**X** = A or V], $Y_{153}\underline{\mathbf{X}}ENMY_{158}$ [**X** = R or H], or $Y_{166}RPVD\underline{\mathbf{X}}Y_{172}$ X = Q or R) can be used to quantify polymorphisms at 136, 154, and 171 when the polymorphism at 172 is Y.[47]

Chymotryptic peptide optimization for MRM analysis. The relevant peptide polymorphisms were chemically synthesized (> 95% purity) by a commercial vendor and then used to optimize the instrument parameters. Each peptide was analyzed by mass

spectrometry to ensure the purity and structure. Instrument parameters were optimized for each natural abundance (^{14}N) peptide (Table S-1, Supporting Information) and applied to the corresponding ^{15}N-labeled internal standard peptide analogs. The sheep peptide containing the amino acid F at position 141 ($M_{132}LGSAMSRP\underline{\mathbf{F}}_{141}$) optimally fragmented to yield the y8 ($G_{134}SAMSRP\underline{\mathbf{F}}_{141}$; most intense), y5 ($M_{137}SRP\underline{\mathbf{F}}_{141}$), and y6 ($A_{136}MSRP\underline{\mathbf{F}}_{141}$) ions. This peptide fragment is analogous to the fragment from the 141 L peptide, as previously reported.[47] The most intense ions from the fragmentation of $Y_{166}RPVDQ\underline{\mathbf{D}}SNQNNF_{178}$ were the y11 ($P_{168}VDQ\underline{\mathbf{D}}SNQNNF_{178}$) and $b12^{2+}$ ($Y_{166}RPVDQ\underline{\mathbf{D}}SNQNN$) ions. The two peptides were quantified using these optimized instrument settings. The other peptides used in this work were previously optimized.[47]

The chymotryptic peptides are distinguishable. We demonstrated that the peptides used to quantify the 136, 154, and 171 polymorphisms did not interfere with the analysis of any of the other peptides and could be distinguished from one another.[47] The precursor ions for the peptides $M_{132}LGSAMSRP\underline{\mathbf{L}}_{141}$ (m/z = 545.79), $M_{132}LGSAMSRP\underline{\mathbf{F}}_{141}$ (m/z = 548.77), and $Y_{166}RPVDQ\underline{\mathbf{D}}SNQNNF_{178}$ (m/z = 798.9) have product ions that are distinct from the product ions of the other peptides. Since these chymotryptic peptides have precursor and product ions that are different from those previously described, all of the chymotryptic peptides present in a sample can be readily distinguished from one another by their unique precursor and product ions.

The peptides used to distinguish between the polymorphisms at position 141, $M_{132}LGSAMSRP\underline{\mathbf{X}}_{141}$ ($\underline{\mathbf{X}}$= L or F), and 154 ($Y_{153}\underline{\mathbf{X}}ENMY_{158}$) ($\underline{\mathbf{X}}$ = R or H), contain methionine residues. Each can be oxidized to the corresponding sulfoxide by various enzymatic and artifactual oxidative processes.[54, 55] The signal from an oxidized

methionine would not be apparent using a method to detect only the unoxidized methionine. The MRM methods for these peptides were modified to include methionine oxidation. The values reported for the peptides are the sum of the unoxidized and oxidized forms of the peptide. The amount of oxidized methionine in the Y_{153}**X**ENMY_{158} (**X** = R or H) peptide was found to be less that 6% of the total PrP^{C} or PrP^{Sc} containing samples. The M_{132}LGSAMSRP**X**$_{141}$ (**X**= L or F) peptides have two methionines, meaning that oxidation can result in four distinct peptides: no methionines oxidized, two with one of the two methionines oxidized, and one with both methionines oxidized. Unfortunately, the high background relative to the signals from the three oxidized peptides precluded an accurate quantitation of the oxidation levels of these peptides. Provided that the amount of PrP^{Sc} is not too low, this method which includes the contribution of oxidized methionines should provide an accurate estimate of the amount of each of these two peptides.

Assessing the interference from background noise with the chymotryptic peptides. Processing and digesting the prion enriched pellet with chymotrypsin yields a large number of molecules. It is possible that some of these molecules may interfere with our subsequent analysis. In order to interfere with this analysis, a molecule would have to have an identical chromatographic retention time and identical precursor and product ions. It was therefore necessary to determine if signals from any of these peptides interfered with signals from other peptides. The synthetic peptides were analyzed to determine if there was overlap with other peptides. The signals from pure chymotryptic peptides do not interfere with the analysis of the other chymotryptic peptides.

It was necessary to determine whether any molecules present in the chymotryptic digestion of a pellet from an uninfected sheep might have signals that would interfere with this analysis. An ultracentrifuge pellet from an uninfected, PrP homozygous ($A_{136}L_{141}R_{154}Q_{171}Y_{172}/A_{136}L_{141}R_{154}Q_{171}Y_{172}$), sheep brain homogenate was digested with chymotrypsin. The chymotryptic digestion of ^{15}N-labeled ($A_{136}L_{141}R_{154}R_{171}Y_{172}$) was added to the chymotryptic digest as the internal standard and the samples were analyzed by this MRM-based method (Figure S-4, Supporting Information). Examination of the chromatograms revealed that none of the signals was above noise. In conclusion, the chymotryptic digest does not produce molecules, other than those expected from the residual PrP^C, which would significantly interfere with the analysis.

Generating stable isotope (^{15}N) incorporated internal standards. Clones expressing relevant rPrP were grown in minimal medium supplemented with $^{15}NH_4Cl$. ^{15}N was incorporated into all of the proteins of *E. coli*, in place of the natural abundance (^{14}N) nitrogens. The isotopic purity of the ^{15}N-labeled peptides was very high (> 99.7%). After the appropriate ^{15}N-labeled rPrP was purified from the clones and digested with chymotrypsin, it yielded a set of ^{15}N-labeled peptides that was used as an internal standard. By analyzing internal standard ^{15}N-labeled peptides with a ^{15}N-based MRM method we determined that none of the signals from a ^{15}N-labeled peptide interfered with the signal of any of the other internal standard ^{15}N-labeled peptides. Furthermore, this analysis showed that the signals from the ^{15}N peptides did not interfere with those of the natural abundance (^{14}N) chymotryptic peptides.

Quantifying peptide amounts in a sample using calibration curves. The quantification of synthetic peptide M_{132}LGSAMSRP**F**$_{141}$ was provided by the vendor. The amounts of Y_{166}RPVDQ**D**SNQNNF$_{178}$ and GQPHGGGW (Supporting Information) were determined by measuring the absorbance at 280 nm of the peptide solutions. Chymotryptic digestion of the ^{15}N-labeled PrP polymorphisms was used to generate the appropriate ^{15}N-labeled internal standard peptides. We prepared two sets of dilutions (10, 20, 50, 500, 1000, or 10,000 attomole/injection) containing either the two peptides with the 141 polymorphism (M_{132}LGSAMSRP**L**$_{141}$ and M_{132}LGSAMSRP**F**$_{141}$) or the one with the 172 polymorphism (Y_{166}RPVDQ**D**SNQNNF$_{178}$) or the peptide from the octarepeat region (GQPHGGGW) and 1 fmol of the synthetic peptide R_{159}YPNQVY$_{165}$. These two solution sets were analyzed by mass spectrometry.

The area of the signals from an optimized ion of one of the four synthetic peptides and ^{15}N-labeled R_{159}YPNQVY$_{165}$ peptide were determined. The area ratios of the dilutions of the synthetic peptides to the fixed amount of ^{15}N-labeled R_{159}YPNQVY$_{165}$ peptide in each dilution were calculated (n=4) for each solution in the two sets (Figures S-5 to S-8, Supporting Information). Calibration curves relating these calculated area ratios to the fixed amount of the ^{15}N-labeled R_{159}YPNQVY$_{165}$ peptide were calculated. The curves were determined to be linear over a >100-fold range with excellent correlation coefficients (> 0.99).

The signal intensity of the M_{132}LGSAMSRP**L**$_{141}$ peptide relative to R_{159}YPNQVY$_{165}$ was determined to be 0.28 ± 0.04 and for M_{132}LGSAMSRP**F**$_{141}$ the signal intensity relative to R_{159}YPNQVY$_{165}$ was determined to be 0.23 ± 0.07. Unfortunately, the signal intensity of Y_{166}RPVDQ**D**SNQNNF$_{178}$ relative to

$R_{159}YPNQVY_{165}$ is approximately 6 fold lower than that of either $Y_{166}RPVD\underline{\mathbf{Q}}Y_{172}$ or $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ relative to $R_{159}YPNQVY_{165}$. This means that a signal for $Y_{166}RPVDQ\underline{\mathbf{D}}SNQNNF_{178}$ may not be observed in samples containing low amounts of PrP obtained from heterozygous animals ($A_{136}L_{141}R_{154}\underline{\mathbf{R}}_{171}\underline{\mathbf{Y}}_{172}/A_{136}L_{141}R_{154}\underline{\mathbf{Q}}_{171}\underline{\mathbf{D}}_{172}$), even though a signal for $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ would be observed (*vide infra*).

Quantifying the amounts of the 171 R and Q polymorphisms relative to the $R_{159}YPNQVY_{165}$ peptide. In a chymotryptic digest of sheep PrP, $Y_{166}RPVD\underline{\mathbf{Q}}Y_{172}$ or $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ are produced in direct proportion to $R_{159}YPNQVY_{165}$. We previously demonstrated the linear relationship between the area ratios of the observed signals from these peptides.[47] A set of six solutions containing 0.5, 1.5, 2.5, 3.5, 4.5 or 5.0 femtomoles of the $Y_{166}RPVD\underline{\mathbf{Q}}Y_{172}$ or $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ and 5 femtomoles of the $R_{159}YPNQVY_{165}$ peptide were prepared and analyzed by our method. The curves (Figure S-9, Supporting Information) were determined to be linear with good correlation coefficients (> 0.920).

The ratios of the signal intensities of $Y_{166}RPVD\underline{\mathbf{Q}}Y_{172}$ or $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ to $R_{159}YPNQVY_{165}$ were determined for two PrP samples. Forty-six samples of classical sheep scrapie ($A_{136}L_{141}R_{154}Q_{171}Y_{172}/A_{136}L_{141}R_{154}Q_{171}Y_{172}$) were digested with chymotrypsin and analyzed by triplicate injections. The area ratio of the signals from the $Y_{166}RPVD\underline{\mathbf{Q}}Y_{172}$ to $R_{159}YPNQVY_{165}$ peptides was determined to be 0.20 ± 0.08. This value was not statistically different ($p > 0.05$) from the value (0.17 ± 0.02) obtained by the injection of pure peptides. In a separate experiment twenty-five samples of ^{15}N-labeled sheep rPrP ($A_{136}L_{141}R_{154}R_{171}Y_{172}$) were digested with chymotrypsin and analyzed by triplicate injections. The area ratio of the signals from the $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ to

$R_{159}YPNQVY_{165}$ peptides was determined to be 0.15 ± 0.04. This value was not statistically different ($p > 0.05$) from the value (0.14 ± 0.01) obtained by the injection of pure peptides.

By using this approach the relative amounts of $A_{136}L_{141}R_{154}R_{171}Y_{172}$ and $A_{136}L_{141}R_{154}Q_{171}Y_{172}$ polymorphisms can be independently determined. This is not necessary when a sample from a heterozygous animal contains both the $A_{136}L_{141}R_{154}R_{171}Y_{172}$ and $A_{136}L_{141}R_{154}Q_{171}Y_{172}$ polymorphisms, since the signal intensities of the $Y_{166}RPVD\mathbf{\underline{Q}}Y_{172}$ and $Y_{166}RPVD\mathbf{\underline{R}}Y_{172}$ peptides are similar (*vide supra*), which means both peptides can be determined independently. It is essential when the heterozygous animal contains $A_{136}L_{141}R_{154}R_{171}Y_{172}$ and $A_{136}L_{141}R_{154}Q_{171}D_{172}$, since the $Y_{166}RPVDQ\mathbf{\underline{D}}SNQNNF_{178}$ is undetectable relative to $Y_{166}RPVD\mathbf{\underline{R}}Y_{172}$ in a sample containing low amounts of PrP. This approach was used to calculate the amount of the 171R polymorphism present in the O-1391 heterozygous animal ($A_{136}L_{141}R_{154}R_{171}Y_{172}$/ $A_{136}L_{141}R_{154}Q_{171}D_{172}$), since the amount of PrP present in the sample was too low to detect the signal from the $Y_{166}RPVDQ\mathbf{\underline{D}}SNQNNF_{178}$ peptide.

Determining the limit of detection for sheep PrP polymorphisms at positions 141 and 172. Two synthetic peptides, $M_{132}LGSAMSRP\mathbf{\underline{F}}_{141}$ and $Y_{166}RPVDQ\mathbf{\underline{D}}SNQNNF_{178}$, were serially diluted and independently analyzed using this MRM method. The signal intensities of the $M_{132}LGSAMSRP\mathbf{\underline{F}}_{141}$ peptide are more intense than that of the $Y_{166}RPVDQ\mathbf{\underline{D}}SNQNNF_{178}$ peptide (Figure S-10, Supporting Information). These results show a limit of detection (LOD; signal ≥ 3x background) of 50 attomoles or less (S/N > 3 x background) for these two peptides. The LOD for $M_{132}LGSAMSRP\mathbf{\underline{F}}_{141}$ and $Y_{166}RPVDQ\mathbf{\underline{D}}SNQNNF_{178}$ peptides are comparable (~50

attomole), but higher than those possessing the R or Q polymorphism at position 171 (Y_{166}RPVD**Q**Y_{172} or Y_{166}RPVD**R**Y_{172}).[47] The peptides spanning the 171 polymorphism were part of the PrP protein containing other polymorphisms at positions 141, 154, 171 or 172. Since the peptides spanning the 171 polymorphisms have lower LODs, and lack methionine, they were used to determine the relative proportions of the PrP polymorphisms present in the each of PrP proteins present in these samples.

Quantifying the amount of PrP^C in a scrapie-infected sample. When transgenic mice expressing different levels of PrP^C are experimentally infected with atypical scrapie, the incubation period of the disease is strongly related to those expression levels.[25, 26] It is important to determine the amount of PrP^C expressed in brain tissue from atypical scrapie-infected and control sheep to determine if the infected sheep have noticeably higher PrP^C expression levels. Ultracentrifugation is a well established method of enriching a prion (> 95%) sample by pelleting the prions present in the sample.[56] This also means that the supernatant contains PrP^C that is largely devoid of prions. We employed a modified version of the Bolton *et al.* method to enrich the prion samples (> 95%).[51, 56] The pellet contains the insoluble PrP^{Sc} and a small amount of residual PrP^C. The supernatant contains the soluble PrP^C and a small amount of PrP^{Sc} (<5%). Each of the atypical scrapie cases and the uninfected control cases were analyzed using this approach.

We used immobilized metal affinity purification to isolate the PrP^C in the ultracentrifugation supernatant and mass spectrometry to quantify the amount of PrP^C present in the sample (Figure S-11, Supporting Information). The supernatant was diluted with one volume of 8 M guanidine hydrochloride (GuCl), allowed to stand for 24

hours at room temperature to inactivate any remaining prions and then loaded on a copper charged IMAC column. After several wash steps, PrP^C was eluted with 250 mM imidazole in 2M GuCl and 5% sarkosyl. The wash solutions and eluant were separately analyzed by mass spectrometry.

A significant portion of PrP^C was present in the column flow through, which suggests that it is missing the copper binding N-terminus and not binding to the copper IMAC column. The area ratio of the signal for the copper binding octarepeat peptide (GQPHGGGW, Supporting Information) to that of $R_{159}YPNQVY_{165}$ was substantially lower (~1/6) in the flow through than in the eluted PrP^C. The ratio of the PrP polymorphisms were the same as those observed in Table 1 in both the flow through and the eluted PrP^C. The truncated PrP^C present in atypical scrapie samples O1359, O1376, O1391, and O1800 was approximately 47 ± 3 %, 32 ± 2 %, 19 ± 2 %, or 22 ± 2 % of the total PrP^C present in these samples, respectively. For the O1256, O1388(s), and O1388(c) samples, the values were < 1%; 11 ± 6 %, 25 ± 3 %, respectively. The differences in the amount of truncated PrP^C present in the control samples may be due to the time, *post mortem*, that they were harvested or differences in the tissue composition. Based on these results the material in the flow through is the previously described N-terminally truncated C1 or C2 form of PrP^C.[57, 58]

The amount of PrP^C present in the supernatant and the relative proportion of each polymorphism was determined and summarized in Table 1. The amount of PrP^C in the samples from infected sheep was variable and comparable, between 3.1 and 5.7 picomole/100 mg brain tissue. The amount of PrP^C detected in the control samples was between 2.2 and 7.2 picomole/100 mg brain tissue. By comparison, the amount of PrP^C/g

present in a whole sheep brain homogenate has been estimated, by Western blot, to be between 3.8 and 4.8 ug/g or between 100 at 130 pmol/g.[59] Differing amounts of PrP^C in these samples are a reflection of varying amounts of PrP^C found in different brain regions of different genotypes of sheep.[60, 61] However, the relative amount of each polymorphism was consistent with equal expression of the two *PRNP* polymorphisms.

In transgenic mice, the progression of atypical scrapie is more strongly influenced by the expression levels of the native PrP^C than is classical scrapie.[25, 26] The results summarized in Table 1 show that the expression levels of PrP^C are similar for all of the samples, which suggests that PrP^C expression levels alone are not responsible for the generation of atypical scrapie in these sheep.

Sample	Amount (Femtomole)	Proportion 171Q	Proportion 171R
O1359	$4.0 \times 10^3 \pm 2 \times 10^2$	0.45 ± 0.04	0.55 ± 0.04
O1376	$5.7 \times 10^3 \pm 2 \times 10^2$	0.48 ± 0.02	0.52 ± 0.02
O1391	$3.3 \times 10^3 \pm 4 \times 10^2$	0.43 ± 0.07	0.57 ± 0.07
O1800	$3.1 \times 10^3 \pm 2 \times 10^2$	0.56 ± 0.05	0.44 ± 0.05
O1256	$2.2 \times 10^3 \pm 1 \times 10^3$	0.53 ± 0.03	0.47 ± 0.03
O1388(s)	$7.2 \times 10^3 \pm 1 \times 10^3$	0.53 ± 0.03	0.47 ± 0.03
O1388(c)	$2.9 \times 10^3 \pm 3 \times 10^2$	0.49 ± 0.04	0.51 ± 0.04

Table 1. Amount of PrP^C present in the supernatant from each sheep sample (n=6). The supernatant is derived from the Bolton procedure (*vide supra*) and reported as femtomoles per 100 mg of brain tissue. The genotype of each animal was determined by sequencing the *PRNP* gene (Table S-2, Supporting Information).

Quantifying the amount of PrP present in and the relative contribution of the PrP polymorphisms to the PrP^{Sc} isolated from a heterozygous animal.[47] The amount of PrP^{C} present in the pellet was noticeably greater in the cerebellum sample than that of the cranial spinal cord of animal O1388. For animal O1256, the amount of PrP in the cranial spinal cord pellet was similar to that of the cerebellum from animal O1388 and similar to that found in a whole brain homogenate from another uninfected animal.[47] The PrP^{C} present in the pellets from uninfected sheep (O1256 and O1388) was less than 5% of the total PrP^{Sc} in the samples O1359, O1376, and O1800, but not O1391 (Table 2). It is also less than 2.5% of the PrP^{C} present in the respective samples (Table 1).

The four sheep infected with atypical scrapie (Table S-2, Supporting Information) were identified during the routine screening for TSEs in small ruminants. The four brainstem samples were homogenized in denaturing detergent and subjected to ultracentrifugation according a modified Bolton *et al.* method.[51, 56] Pellets from these four samples (n=4) were denatured and then analyzed by a mass spectrometry-based method to determine the amount of total PrP^{Sc}.[47] PK digestion of these atypical scrapie containing pellets would remove the portion of the PrP protein containing the polymorphisms at positions 154, 171, and 172, so these samples were not digested with PK. The result of this analysis is shown in Table 2.

The amount of PrP^{Sc} in the infected samples varied among the samples. The amounts in samples O1359, O1376, and O1800 were sufficiently large that any residual PrP^{C} in the sample would be a minor contribution (0.3 to 6 %). Sample O1391 contained such a low amount of PrP^{Sc} that the contribution from residual PrP^{C} was significant

(between 5 and 50%). Since variations in the amount of PrP^{Sc} in a sample may be due to the stages of the disease or distribution of the PrP^{Sc} in the source tissue, the cause of the low amount of PrP^{Sc} in sample O1391 is unknown.

When heterozygous sheep (VRQ/ARR) are infected with classical scrapie, the PrP^{Sc} is composed entirely of VRQ and not ARR PrP.[48, 49] All of the atypical scrapie samples contained a significant amount of the ARR polymorphism in the PrP^{Sc}, which is analogous to what is observed when heterozygous (VRQ/ARR) sheep are infected with BSE.[49] Since atypical scrapie is thought to originate spontaneously and has a long incubation period, these comparatively small differences suggest that the $A_{136}L_{141}R_{154}R_{171}Y_{172}$, $A_{136}L_{141}H_{154}Q_{171}Y_{172}$, and $A_{136}F_{141}R_{154}Q_{171}Y_{172}$ propagate at a similar, albeit slightly different rate, with $A_{136}L_{141}H_{154}Q_{171}Y_{172}$, and $A_{136}F_{141}R_{154}Q_{171}Y_{172}$ replicating slightly faster than the $A_{136}L_{141}R_{154}R_{171}Y_{172}$. In sample O1391, $A_{136}L_{141}R_{154}R_{171}Y_{172}$ replicates slightly faster than $A_{136}L_{141}R_{154}Q_{171}D_{172}$. In classical scrapie, positions 154 and 171 are present in the PK-resistant prion core (amino acids ~90-231), but are not part of the PK resistant core of atypical scrapie (amino acids ~90-150).[4, 34-36, 62] Even though positions 171 and 154 are not part of the PK-resistant core of atypical scrapie, they still influence the propagation of atypical scrapie, but to a lesser extent than is observed in classical scrapie.

Sample	Amount (Femtomole)	Proportion 171Q	Proportion 171R
O1359	$1.6 \times 10^3 \pm 5 \times 10^2$	0.57 ± 0.04	0.43 ± 0.04
O1376	$2.9 \times 10^3 \pm 3 \times 10^2$	0.57 ± 0.04	0.43 ± 0.04
O1391	$0.1 \times 10^3 \pm 3 \times 10^2$	0.3 ± 0.3	0.7 ± 0.3
O1800	$1.5 \times 10^3 \pm 1 \times 10^2$	0.61 ± 0.07	0.39 ± 0.07
O1256	$0.1 \times 10^3 \pm 2 \times 10^2$	0.5 ± 0.3	0.5 ± 0.3
O1388(s)	$0.005 \times 10^3 \pm 2$	N.D	N.D.
O1388(c)	$0.07 \times 10^3 \pm 2 \times 10^2$	0.65 ± 0.2	0.35 ± 0.2

Table 2. Tabular summary of the amount of PrP present in each sample pellet. The pellet is derived from the Bolton procedure. The amount present in the pellet is reported in femtomoles per 100 mg of brain tissue. The genotype was determined by sequencing the *PRNP* gene (Table S-2, Supporting Information). N.D. not determined.

Conclusions

We quantified the relative amount of PrP polymorphisms present in atypical PrP^{Sc} from heterozygous sheep. Significant amounts of both polymorphisms at positions 141, 154, 171 and 172 are present in these heterozygous animals. Furthermore, we quantified the full length and the C1 forms of PrP^{C} present in sheep infected with atypical scrapie. We determined the ratio of the PrP polymorphisms present in the PrP^{C} and PrP^{Sc} of the same sample and estimated the expression levels of the full length and C1 forms of

PrP^C in the samples. We used the linear relationship between the area ratios of the signals from the peptides $Y_{166}RPVD\underline{\mathbf{Q}}Y_{172}$ or $Y_{166}RPVD\underline{\mathbf{R}}Y_{172}$ to $R_{159}YPNQVY_{165}$ to calculate the relative amounts of each polymorphism present in the sample. This is relevant when a polymorphism, such as D_{172}, prevents quantification of one polymorphism.

In transgenic animals, the progression of atypical scrapie is dependent upon the expression levels of PrP^C.[25, 26] We showed that the expression levels of each PrP^C polymorphism in both infected and control heterozygous animals were similar. In addition, the overall expression of PrP^C was similar for the control animals and those infected with atypical scrapie. This supports a hypothesis that atypical scrapie is not caused by PrP^C overexpression in afflicted animals. Excessive production of the truncated form of PrP^C (C1) is associated with the resistance of homozygous $A_{136}L_{141}R_{154}R_{171}Y_{172}$ sheep to classical scrapie.[63] In these atypical scrapie samples C1 was present in significant amounts. Furthermore, both polymorphisms of C1 were found in equal amounts. These results indicate that atypical scrapie is not caused by an overexpression of PrP^C, nor does it appear related to the presence of C1.

The overall progression of atypical scrapie may be inherently slower in heterozygous animals than in homozygous animals. In heterozygous animals, each PrP^C polymorphism is expressed at half the level of the total amount of PrP^C. Since the progression of atypical scrapie is strongly dependent upon the levels of PrP^C, heterozygous animals should have longer incubation periods for atypical scrapie than homozygous animals since each PrP^{Sc} polymorphism would be replicating in an animal

expressing only half the level of the total PrP^C substrate. Experimental transmission of atypical scrapie to heterozygous sheep has not yet been reported.

A spontaneous prion disease begins with a single prion which replicates during an extended incubation period, lasting at least several months.[7] This study demonstrated that there are significant amounts of both polymorphisms, including $A_{136}L_{141}R_{154}R_{171}Y_{172}$, in atypical scrapie, which implies that their relative rates of replication are very similar; if it were not the case, one polymorphism would outcompete the other. By comparison, heterozygous sheep ($V_{136}L_{141}R_{154}Q_{171}Y_{172}/A_{136}L_{141}R_{154}R_{171}Y_{172}$) infected with classical scrapie have very little of the $A_{136}L_{141}R_{154}R_{171}Y_{172}$ polymorphism in their PrP^{Sc}.[48] Our results show that, with atypical scrapie, relative to the $A_{136}L_{141}R_{154}R_{171}Y_{172}$ polymorphism, the $A_{136}L_{141}R_{154}H_{171}Y_{172}$ and $A_{136}F_{141}R_{154}Q_{171}Y_{172}$ polymorphisms replicate only slightly faster and the $A_{136}L_{141}R_{154}Q_{171}D_{172}$ polymorphism replicates only slightly slower. This result predicts that atypical scrapie should be widely distributed among animals with differing PrP genotypes (with A at position 136), which was seen in a large study of European atypical scrapie cases by genotype.[20]

Supporting Information

Table of optimized instrument parameters, text with supporting experimental procedures, animal genotypes, immunohistochemistry images, Western blots, figures of calibration curves, and figure for limit of detection (PDF)

Notes

The authors declare no competing financial interests.

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