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Significant differences in incubation times in sheep infected with bovine spongiform encephalopathy result from variation at codon 141 in the *PRNP* gene

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The susceptibility of sheep to prion infection is linked to variation in the *PRNP* gene, which encodes the prion protein. Common polymorphisms occur at codons 136, 154 and 171. Sheep which are homozygous for the A₁₃₆R₁₅₄Q₁₇₁ allele are the most susceptible to bovine spongiform encephalopathy (BSE). The effect of other polymorphisms on BSE susceptibility is unknown. We orally infected ARQ/ARQ Cheviot sheep with equal amounts of BSE brain homogenate and a range of incubation periods was observed. When we segregated sheep according to the amino acid (L or F) encoded at codon 141 of the *PRNP* gene, the shortest incubation period was observed in LL₁₄₁ sheep, whilst incubation periods in FF₁₄₁ and LF₁₄₁ sheep were significantly longer. No statistically significant differences existed in the expression of total prion protein or the disease-associated isoform in BSE-infected sheep within each genotype subgroup. This suggested that the amino acid encoded at codon 141 probably affects incubation times through direct effects on protein misfolding rates.

INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a member of a group of fatal neurodegenerative diseases called transmissible spongiform encephalopathies (TSEs). Natural hosts of infection for the BSE agent are cattle and humans, but sheep can also be infected experimentally. Other examples of TSEs in animals are classical and atypical scrapie in sheep and goats and chronic wasting disease (CWD) in deer. It is widely believed that TSEs are caused by the misfolding of a host-expressed glycoprotein, called the prion protein (PrP^C). The pathological form of PrP manifest in diseased individuals is termed PrP^{Sc}. Characteristic hallmarks of TSE disease include vacuolation in neural tissues and deposition of PrP^{Sc} in brain and lymphoid tissue. Typically, the time between infection and the demonstration of clinical signs and ultimately death of an affected individual is long, in many cases years.

The prion protein is encoded by the *PRNP* gene, which in sheep is highly polymorphic (Baylis & Goldmann, 2004; Goldmann, 2008). Common polymorphisms occur at positions 136, 154 and 171 and are responsible for encoding the

amino acids alanine/valine (A/V), arginine/histidine (R/H) and glutamine/arginine/histidine (Q/R/H), respectively. The strain of the infecting agent and the route of infection, in combination with the amino acid expressed at these codons, modulate both the relative susceptibility to TSE infection and associated incubation periods. For example, the V₁₃₆R₁₅₄Q₁₇₁ allele (VRQ) is associated with a higher susceptibility to classical scrapie, compared with the ARR allele that is associated with increased resistance and lengthened incubation periods. Similarly, the ARQ allele is associated with susceptibility to experimental BSE infection in sheep compared with the ovine VRQ and ARR alleles. Additional *PRNP* coding region polymorphisms have been identified, though often they occur at low frequencies, which make it difficult to assess their association with TSE susceptibility. Nonetheless, polymorphisms such as M112T (Laegreid *et al.*, 2008; Saunders *et al.*, 2009), P168L (Goldmann *et al.*, 2006) and N176K (Vaccari *et al.*, 2007, 2009) all appear to be associated with protection against BSE or classical scrapie infection. In contrast, when phenylalanine (F) is substituted for the wild-type amino acid leucine (L) at codon 141 of ovine *PRNP* (Bossers *et al.*, 1996) this change is associated with an increased susceptibility to atypical scrapie (Moum *et al.*, 2005). To date,

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there has been no reported association of this polymorphism with susceptibility to BSE. Evidence would suggest that sheep in the UK flock have not been infected with BSE during the cattle BSE epidemic of the 1980s/1990s; however, it is important to determine the role of additional amino acids (out with codons 136, 154 and 171) to assess susceptibility of sheep to potential emerging TSE infections. Following oral infection with BSE, we show that FF₁₄₁ homozygous and LF₁₄₁ heterozygous sheep have prolonged incubation periods compared with LL₁₄₁ homozygous sheep.

RESULTS AND DISCUSSION

Of the 39 sheep challenged with BSE brain homogenate, four were confirmed as negative for BSE infection at the time of euthanasia using both immunohistochemistry and Western blotting (McCutcheon *et al.*, 2011). Of the other 35 BSE-infected sheep, 32 were confirmed as having BSE and reached the clinical phase of disease with incubation periods ranging from 534 to 1593 days and a mean incubation period of 939 ± 316.1 days (\pm SD). The remaining three sheep infected with BSE are alive and classed as survivors, though the pathological status of these animals is at present unknown. None of the negative controls developed disease. Although all sheep were of the ARQ/ARQ genotype, the wide range of incubation times seen in the confirmed BSE cases prompted us to consider whether this could be explained by additional genetic effects in the *PRNP* gene. Further analysis of this gene revealed variation in the amino acid encoded at position 141 (L to F substitution). Sheep were grouped accordingly to whether they were LL₁₄₁, FF₁₄₁ or LF₁₄₁ as shown in Table 1. Fig. 1(a) shows a graphical representation of incubation periods for all BSE-infected sheep after subdividing them by codon 141 genotype. The median incubation periods of the cases that succumbed to BSE were 603 days post-inoculation (p.i.) for LL₁₄₁ sheep ($n=11$), 840 days p.i. for FF₁₄₁ sheep ($n=8$) and 1231 days p.i. for LF₁₄₁ sheep ($n=13$). Standard Kaplan–Meier analysis of differences in incubation and survival times between the sheep in each genotype was carried out. This revealed highly significant differences in

median incubation times in BSE-infected sheep between all three genotype subgroups ($\chi^2=42.3$, $P<0.001$, Fig. 1b). In the same analyses, the four sheep that were culled but confirmed BSE negative were censored and the three sheep that are still alive were right-censored.

To investigate whether incubation period differences correlate with variations in PrP levels, we analysed homogenates, from the medulla, by Western blotting. Fig. 2(a) shows a representative immunoblot of PrP protein profiles in these homogenates, prepared from BSE-infected sheep, following detergent extraction and treatment either without (lanes 1–6, equating to total PrP protein) or with proteinase K (PK, $50 \mu\text{g ml}^{-1}$), respectively (lanes 7–12, equating to PK-resistant PrP^{Sc}). For each genotype subgroup, results from two animals are shown. The protein profile shown in lanes 7–12 confirms BSE infection by the presence of PK-resistant PrP^{Sc}. The immunoblot of the same samples, though not treated with PK (in lanes 1–6) were also probed with an anti-tubulin antibody as a loading control (Fig. 2b). Densitometric analysis (Fig. 3) identified no statistically significant differences ($P>0.05$, Tables 2 and 3) between genotype groups neither in terms of total PrP nor in disease-associated PrP^{Sc} deposited in the medulla of the brain at the clinical end-point in BSE-infected sheep (Fig. 2a). Thus, the increased incubation times in FF₁₄₁ and LF₁₄₁ sheep relative to LL₁₄₁ sheep could be caused by either delayed routing of infection from the gut to the central nervous system or through a direct effect of the amino acid change on the ability of PrP^C to misfold.

The importance of amino acid substitutions in the PrP protein for directing susceptibility to TSEs has long been recognized (Andréoletti *et al.*, 2006; Foster *et al.*, 2001; Goldmann *et al.*, 2006; Houston *et al.*, 2003). Polymorphisms in the *PRNP* gene modulate not only susceptibility to prion disease but also alter incubation times and may account for some of the differences in pathology typically observed in affected individuals (Barron *et al.*, 2005). In sheep, the A136V mutation is associated with increased susceptibility to sheep scrapie, whereas Q171R is associated with resistance (Goldmann *et al.*, 1991, 1994; Hunter *et al.*, 1993). Indeed, genetic variation in the *PRNP* gene and the

Table 1. Infection status of sheep of different PrP genotypes at codon 141 following oral BSE inoculation

Amino acids at codon 141 are represented by leucine (L) or phenylalanine (F). The mean and median incubation period (ip) recorded for the confirmed BSE-infected sheep is shown in days p.i. Survival periods of surviving sheep were calculated in days p.i. and as of November 2011. NA, Not applicable.

Genotype at codon 141	No. sheep per genotype group	No. BSE +ve sheep	Mean ip (\pm SD) for BSE +ve sheep (days p.i.)	Median ip for BSE +ve sheep (days p.i.)	No. BSE –ve sheep	No. of survivors	Survival period (days p.i.)
LL	12	11	625 ± 110.5	603	1	0	–
FF	9	8	858 ± 131.6	840	0	1	1848
LF	18	13	1253 ± 190.3	1231	3	2	1813, 1883
Total	39	32	NA	NA	4	3	–

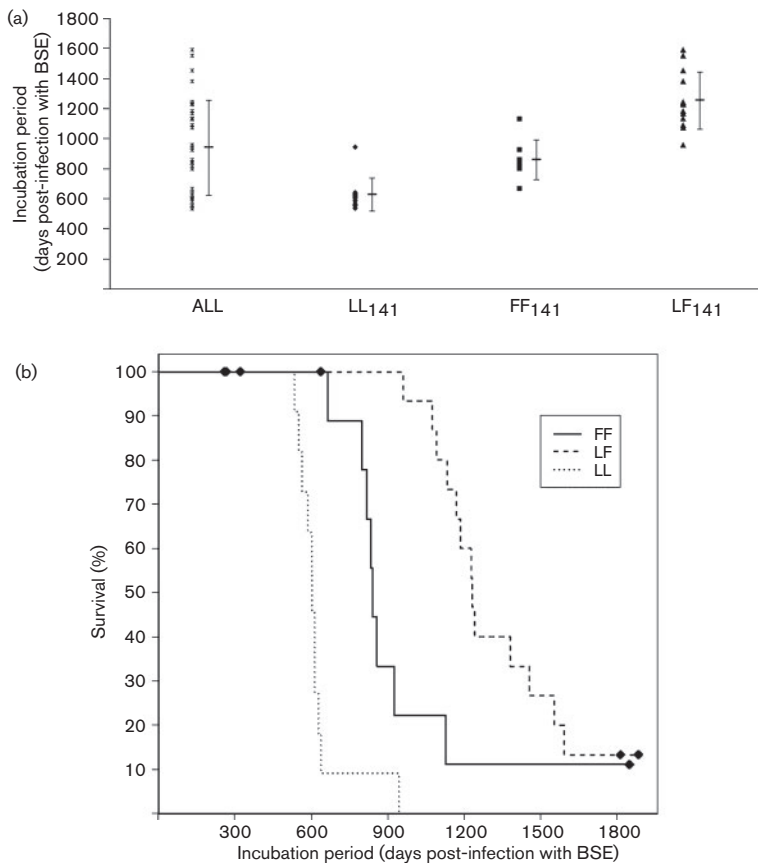


Fig. 1. Incubation period in sheep confirmed as having BSE is modulated by polymorphisms at codon 141. (a) Shows the incubation period of all sheep confirmed as having BSE infection (ALL) and codon 141 genotype subgroups (based on amino acid sequence at codon 141) that were confirmed as having BSE. The mean incubation period of each group is indicated by the solid bar (\pm SD). (b) Represents a Kaplan-Meier survival curve, showing the survival profile of the confirmed BSE cases. The four BSE-negative sheep at the time of euthanasia and the three surviving sheep were censored from the analysis and are indicated by the black diamonds.

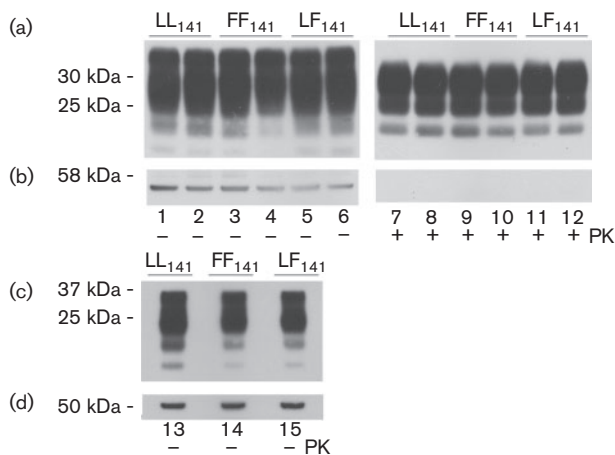


Fig. 2. Confirmation of BSE infection in challenged sheep using Western blotting. (a) Immunoblots for PrP of detergent-extracted, brain homogenates prepared from six BSE-infected sheep each of different genotype subgroups at codon 141. Samples shown in lanes 1–6 were not treated with the enzyme PK. Equivalent samples treated with PK are shown in lanes 7–12. (b) The same samples from (a) probed with anti-tubulin antibody as a loading control. (c) Immunoblot of detergent-extracted brain homogenates prepared from three uninfected, control sheep (one of each codon 141 genotype subgroup). (d) The same samples from (c) probed with anti-tubulin antibody as a loading control.

associated outcomes in relation to disease resistance were used to devise the National Scrapie Plan for the control and eradication of classical scrapie within UK and European flocks (Dawson *et al.*, 1998; Detwiler & Baylis, 2003). Following the emergence of atypical scrapie (Benestad *et al.*, 2003) and its association to the L141F polymorphism (Benestad *et al.*, 2003; Moum *et al.*, 2005) it became apparent that predictions regarding TSE susceptibility may have to be widened. Until now, a significant association with

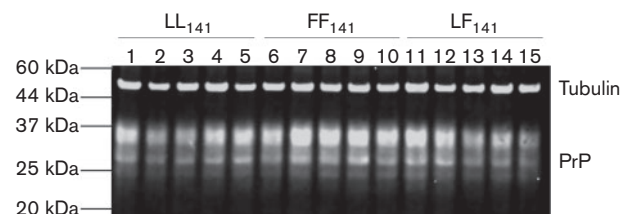


Fig. 3. Representative Western blot used for densitometric comparison of PK-treated and untreated BSE-infected and -uninfected control brain homogenates. Samples in lanes 1–5 are independent non-PK-treated brain homogenates from five individual BSE-infected sheep with LL genotypes, lanes 6–10 samples from five sheep with the FF genotype and lanes 11–15 from five sheep with the LF genotype. Samples were probed for anti-tubulin and prion protein (a).

Table 2. Comparison of *P*-values from densitometric analysis of normalized, non-PK-treated and PK-treated samples in BSE-infected sheep

PrP^C (non-PK-treated brain homogenates) and PrP^{Sc} (PK-treated brain homogenates) from five sheep with confirmed BSE-infections were analysed using SDS-PAGE and Western blotting in three independent experiments. Each sample was repeated a minimum of two times for each genotype. A two-tail Student's *t*-test with unequal variance was carried out on normalized PrP^C expression for all three genotypes and also for PK-treated samples.

Genotype group comparison	<i>P</i> -value
Non-PK-treated samples	
LL v LF	0.5951
LL v FF	0.5279
LF v FF	0.9437
PK-treated samples	
LL v LF	0.9548
LL v FF	0.8141
LF v FF	0.2017

disease of the phenylalanine at codon 141 was limited to atypical scrapie, although an association with classical scrapie resistance has been suggested. It is clear that the role of additional amino acids in disease susceptibility needs to be considered to allow accurate predications of TSE susceptibility. In this report, we have demonstrated for the first time an effect of the ovine L141F polymorphism on susceptibility to BSE infection.

Although sheep of all three codon 141 genotypes have succumbed to oral BSE exposure, the mean incubation period of LF₁₄₁ heterozygotes was approximately twice that of the wild-type LL₁₄₁ homozygotes. Mechanistically, it is not clear why incubation periods vary so dramatically as a function of codon 141 genotype, but a number of possible explanations exist. We can exclude a significant effect of gene dosage (Manson *et al.*, 1994) since Western blotting in

Table 3. Comparison of the mean levels of PrP^C from densitometric analyses performed on uninfected control animals

Replicate samples from uninfected control brain homogenates (PrP^C), from a single sheep in each genotype group, were analysed using densitometric analysis, to allow for an estimation of the variability in PrP^C levels in sham-infected sheep. The value shown for each genotype group represents the relative densitometric intensity measured corresponding to PrP^C in the non-PK-treated tissue samples.

Genotype	Densitometric intensity (relative units)
LL	1.99
FF	1.97
LF	1.87

uninfected control sheep (Fig. 2c and d lanes 13–15, respectively) has shown that the expression of PrP^C in the three codon 141 genotypes is similar, although we were unable to perform statistical analysis due to the limited number of animals for study (Table 3). It is conceivable, therefore, that the increased incubation periods in F₁₄₁ carriers may result from processes involving formation of PrP^{Sc}. It is likely that the amino acid change has a direct effect on the ability of PrP^C to convert to PrP^{Sc} during disease, since it has been shown that PrP^C containing phenylalanine at codon 141 converts less efficiently to PrP^{Sc} when compared with the wild-type (L₁₄₁) counterpart in an *in vitro*-conversion assay seeded with classical scrapie isolate (Bossers *et al.*, 2000). Whilst the infectious agent used in the *in vitro* study was scrapie rather than BSE, other work has shown that cell-free conversion assays replicate the effect of amino acid substitutions across multiple TSE strains. Hence, it is reasonable to suggest that such an experiment seeded with BSE would also show differential conversion of L₁₄₁ and F₁₄₁ proteins (Kirby *et al.*, 2006).

Assuming that the cattle BSE inoculum is trafficked across the intestinal epithelium equally in each genotype of sheep, differential conversion of the two proteins in our experimental model could occur at one of two subsequent phases of disease, as shown schematically in Fig. 4. Firstly, the exogenous cattle PrP^{Sc} converts endogenous ovine PrP^C to ovPrP^{Sc} and this initial cross-species conversion process

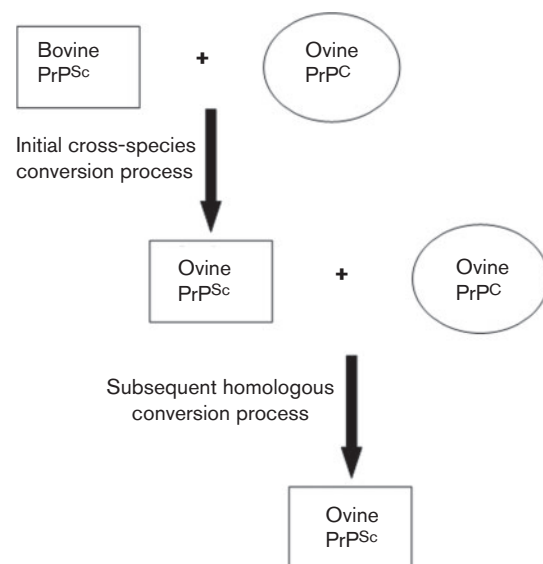


Fig. 4. Schematic depicting the protein-conversion process as two sequential steps. The first step involves the conversion of endogenous ovine PrP^C by the incoming bovine PrP^{Sc} from the inoculum. The efficacy of this reaction may vary with codon 141 genotype, but this step will necessarily be time constrained as the PrP^{Sc} in the initial inoculum is 'diluted out' by the growing levels of ovine PrP^{Sc}. The second step involves homologous conversion of ovine PrP^C by the newly formed ovine PrP^{Sc}.

may be affected by the amino acid at codon 141 in the ovine PrP^C. Alternatively, after this stage the newly formed ovPrP^{Sc} converts more ovPrP^C to ovPrP^{Sc} – a homologous-conversion process. Based on these steps, several scenarios have been modelled, assuming different conversion efficiencies in both the cross-species and homologous-conversion reactions (Fig. 5a–c). These models are extremely simplistic but show neatly how variation in different conversion rates can produce similar outcomes and model the quasi-exponential increase in PrP^{Sc} levels with time. In our experiment, sheep were infected with cattle BSE and the cross-species conversion of PrP^C to PrP^{Sc} may proceed more efficiently for L₁₄₁ protein than for F₁₄₁, leading to higher initial levels of PrP^{Sc} immediately after infection. In such a scenario, homologous-conversion reactions may proceed at equal rates, but the disease process in F₁₄₁-carrying sheep will always trail that in L₁₄₁ sheep (Fig. 5a). Alternatively, the incoming cattle inoculum may convert L₁₄₁ and F₁₄₁ proteins equally, but the subsequent homologous conversion of sheep proteins may proceed at different rates depending on codon 141 (Fig. 5b). Both scenarios can account for the observed incubation times and similar levels of PrP^{Sc} upon euthanasia, but it should be noted that these possibilities are not mutually exclusive. F₁₄₁ protein may convert less efficiently when catalysed by cattle PrP^{Sc} from the incoming inoculum as well as by sheep PrP^{Sc} during the homologous phase of disease (Fig. 5c).

It is noteworthy that heterozygous sheep have the longest incubation periods and, presumably, the least efficient protein conversion. It has continually been hypothesized that this is a result of two distinct misfolding reactions occurring *in vivo*, one involving each protein, and that each proceeds at half the rate as in homozygous animals because only half the substrate exists. Such a scenario has been shown previously in studies of other polymorphisms in the ovine *PRNP* gene. Morel *et al.* used mass spectrometric methods to distinguish PrP^{Sc} encoded by different *PRNP* allotypes and showed that the protein allele associated with susceptibility to scrapie was converted preferentially, resulting in its relative over-representation in pools of PrP^{Sc} (Morel *et al.*, 2007). Likewise, Jacobs *et al.* used antibodies that specifically recognized different ovine PrP^C variants to show that the PK-resistant material in ARR/VRQ sheep affected with scrapie was predominantly from the VRQ allele (Jacobs *et al.*, 2011). This effect is not specific to sheep; in humans heterozygote for a *PRNP* gene carrying a proline to leucine mutation at codon 102, associated with Gerstmann–Straussler–Scheinker disease, the PrP^{Sc} that is deposited during disease appears predominantly to have emanated from the mutated allele (Tagliavini *et al.*, 1994). Almost two decades ago, it was noted that PrP^C, which cannot readily be converted into PrP^{Sc}, can have a dominant-negative effect on conversion of a more susceptible variant in cell culture. Although it is still not clear exactly how such an effect is mediated mechanistically, it seems likely that non-converting PrP^C molecules can bind to PrP^{Sc} aggregates without undergoing conversion themselves, thereby blocking binding sites

for PrP^C that is conversion competent. In any case, our data are consistent with more rapid formation of PrP^{Sc} from the L141 allele in BSE-infected sheep, compared with the F141 allele, but we are currently unable to distinguish the two protein variants using *in vitro* tests so cannot confirm whether or not this is true.

Finally, the confirmation of similar levels of PrP^{Sc} in the brains of sheep at clinical end-point from all three genotypes is compatible with equal conversion rates for the two proteins only if it is assumed that sheep carrying the F₁₄₁ alleles experience delayed transit of disease from gut to brain. Thus, it is possible that the route of infection plays a key role in determining the effect of codon 141 on TSE susceptibility.

METHODS

Ethical statement. All animal work was reviewed and approved by the ethical review panel at The Institute for Animal Health and The Roslin Institute and conducted under the authority of the Home Office Project Licences (references: 30/2282 and 60/4143, respectively).

***In vivo* experiments and data analysis.** As part of our on-going BSE blood transfusion experiments, 39 Cheviot sheep (ARQ/ARQ genotype and derived from a scrapie-free flock) were orally infected with 5 g BSE brain homogenate (Houston *et al.*, 2008; McCutcheon *et al.*, 2011). The inoculum was sourced from the Veterinary Laboratory Agency (VLA) (now known as Animal Health Veterinary Laboratory Agency) and was prepared from brainstem from approximately 100 cows confirmed as having BSE (inoculum reference SE1909/BBP-2). Five groups of eight sheep were infected typically at monthly intervals; the same batch was used to inoculate each sheep. Ten genotype-matched sheep were orally challenged with the same amount of uninfected (non-BSE exposed) bovine brain homogenate (from a single animal) at similar time intervals as the infected cohort. This inoculum was sourced from the VLA (inoculum reference PG1534/05). Following challenge, animals were monitored for clinical signs associated with BSE infection and culled at defined clinical end-points. Non-BSE challenged controls were monitored in the same way and culled at pre-determined time points. For the purposes of data analysis, an experimental end date of November 2011 was assigned. At this point, three BSE-infected sheep were alive with survival periods greater than or equal to 1813 days. Incubation periods from sheep confirmed as having BSE and survival times for animals either alive at the end of the experiment or animals that were negative for BSE upon euthanasia were recorded.

Biochemistry. BSE infection was confirmed by analysis of brain and peripheral lymphoid tissues for the presence of disease-associated PrP using both Western blotting (McCutcheon *et al.*, 2005) using the antibody BC6 (0.1 µg ml⁻¹) and/or immunohistochemistry using antibodies IH9/BG4 (1 µg ml⁻¹, BG4; TSE Resource Centre, The Roslin Institute) by using methods described previously (McCutcheon *et al.*, 2011). BC6 and IH9 were generated in The Roslin Institute against a truncated form of recombinant ovine PrP (ARQ genotype). Immunoblots were also probed with the anti-tubulin antibody alpha Ab-2 (0.01 µg ml⁻¹; Neomarkers).

Densitometric analysis. Western blots of PK-treated and -untreated samples were carried out as described above using the anti-prion mouse monoclonal, BC6, and anti-tubulin antibody. Densitometric analysis was carried out as per manufacturer's instructions (Li-Cor).

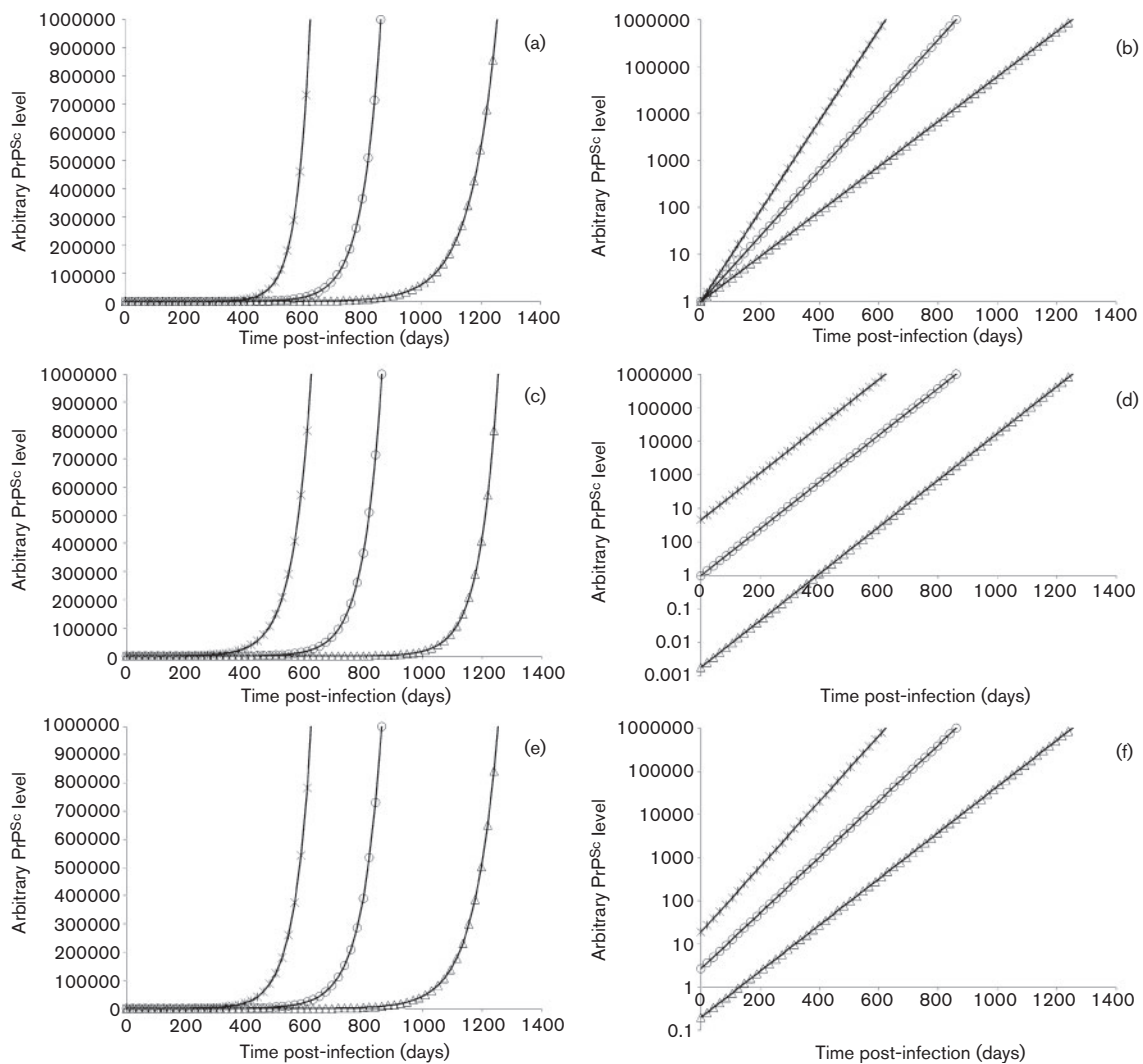


Fig. 5. Simplistic, representative models of exponential increase of PrP^{Sc} caused by the homologous-conversion reaction. An exponential equation of the form $[\text{ovPrP}^{\text{Sc}}]_t = A \cdot k^{(t/T)}$ has been used, where the concentration of ovPrP^{Sc} at time (t) is a function of the variables (A), representing the starting level of ovine PrP^{Sc}, as a proxy for the efficiency of the cross-species conversion reaction, (k) the rate of increase of the concentration and T , the period over which k is measured. The different models assume different starting levels of ovine PrP^{Sc} (in other words, the cross-species generation of ovine PrP^{Sc} by bovine PrP^{Sc} proceeds at differing efficiencies) or different ongoing rates of homologous conversion of ovine PrP^C to ovine PrP^{Sc}. In each case, starting levels and rates have been allowed to vary to bring PrP^{Sc} levels to 10^6 (defined arbitrarily as the amount of PrP^{Sc} in brain at clinical end-point) at the mean incubation time that was determined empirically for each genotype grouping. (a) Equal starting levels of ovine PrP^{Sc} (hence equal cross-species conversion efficiency) but unequal homologous-conversion rates. (b) Different initial ovine PrP^{Sc} levels (hence different efficiencies of cross-species conversion), but equal homologous conversion for each genotype (c) different initial ovine PrP^{Sc} levels and homologous-conversion rates. For each condition, standard graphs of PrP^{Sc} levels are similar, but clear differences can be seen on logarithmic plots. Crosses (X) represent sheep with the LL genotype, circles (O) represent sheep with the FF genotype and triangles (Δ) represent sheep with the LF genotype.

Briefly, protein samples were transferred onto PVDF membrane (Immobilon-P; Millipore), blocked in Odyssey blocking buffer (Li-Cor) for 30 min, primary antibodies were diluted in 0.5% Tween-20 in blocking buffer and were incubated overnight at 4 °C. Blots were washed with 0.1% Tween-20 in PBS, before being incubated with 1:20 000 IRDye 800CW-conjugated goat (polyclonal) anti-mouse IgG (Li-Cor, 926-32210) in 0.01% SDS, 0.5% Tween-20 in PBS. After further washes, blots were visualized on an Odyssey Infrared Imager

and densitometric analysis using the propriety software Image Studio 2. Five animals with confirmed BSE-infections were studied in three independent experiments with each sample repeated a minimum of two times for each genotype. A two-tail Student's t -test with unequal variance was carried out on normalized PrP^C expression for all three genotypes and also for PK-treated samples. In some cases, and for confirmatory purposes, gels and subsequent densitometric analysis of selected samples were repeated a second time using five animals and

two independent samples from each animal. In addition, replicate samples from uninfected control brain homogenates, from a single sheep in each genotype group, were also analysed. This was to make estimates of the variability in PrP^C levels in sham-infected sheep.

PRNP genotyping. PRNP genotyping was performed on PCR-amplified DNA fragments generated from genomic DNA that was extracted from tissue samples collected at post-mortem. A small piece of tissue (≤ 1 mg) was added to 400 μ l of a 1:1 mixed buffer of PBS and TLB [1% (w/v) SDS, 1 mM EDTA, pH 8.0, 10 mM Tris, pH 8.0 and 0.3 M sodium acetate]. To this, 200 μ l PK (20 mg ml⁻¹; Qiagen) was added, mixed and incubated at 37 °C for 1–5 h or overnight. Protein was removed by phenol/chloroform extraction. When tissue was totally dissolved, 400 μ l equilibrated phenol (pH 8.0) and 400 μ l chloroform were added, mixed and spun at 14 000 r.p.m. (20 800 rcf) for 5 min. The upper phase was transferred to a new tube and the extraction repeated. The upper phase was then precipitated following a standard ethanol DNA precipitation protocol. The DNA was dissolved in water or TE buffer (pH 7.5) at a suitable concentration, usually in 100 μ l.

PCR amplification was performed using Sigma JumpStart REDTaq DNA polymerase and buffers, 200 μ M (each) dNTPs (Roche) and 0.4 μ M of each oligonucleotide primer Sigma-Aldrich. Oligonucleotides for the PCR were PS-141d (5'-GGAATGTGAAGAACATTTAT-GACCTAGAAT-3') and PS+109u (5'-CAAGAGAGAAGCAAGA-AATGAGACA-3').

PCR conditions were as follows: one incubation for 3 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 61 °C and 1 min at 72 °C. A final elongation step of 10 min at 72 °C concluded PCR. The PCR products were purified by an addition of 15 μ l activated charcoal suspension (0.8 g of activated charcoal, particle size 100–400 suspended in 50 ml of dH₂O) and incubation at room temperature for at least 10 min. After centrifugation at 12 000 g for 5 min to pellet the charcoal, 1–3 μ l aliquots of the supernatant were taken to sequence the PCR fragments directly in the upstream direction with oligonucleotide PS+50u (5'-CCCCAACCTGGCAAAGATTAAGA-3') using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were purified by ethanol/EDTA precipitation as per the manufacturer's protocol and then run on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Sequences were analysed manually.

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