Protective effect of the T112 PrP variant in sheep challenged with bovine spongiform encephalopathy

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Sheep with an ARQ/ARQ PRNP genotype at codon positions 136/154/171 are highly susceptible to experimental infection with bovine spongiform encephalopathy (BSE). However, a number of sheep challenged orally or intracerebrally with BSE were clinically asymptomatic and found to survive or were diagnosed as BSE-negative when culled. Sequencing of the full PRNP gene open reading frame of BSE-susceptible and -resistant sheep indicated that, in the majority of Suffolk sheep, resistance was associated with an M112T PRNP variant (TARQ allele). A high proportion (47 of 49; 96 %) of BSE-challenged wild-type (MARQ/MARQ) Suffolk sheep were BSE-infected, whereas none of the 20 sheep with at least one TARQ allele succumbed to BSE. Thirteen TARQ-carrying sheep challenged with BSE are still alive and some have survival periods equivalent to, or greater than, reported incubation periods of BSE in ARR/ARR and VRQ/VRQ sheep.

Bovine spongiform encephalopathy (BSE) belongs to a group of diseases known as transmissible spongiform encephalopathies (TSEs) or prion diseases, which include scrapie in sheep and goats and Creutzfeldt–Jakob disease (CJD) in humans. BSE was first identified in cattle in the UK in 1985 (Wells et al., 1987) and has since been reported in at least 25 countries (http://www.oie.int/Eng/Info/en_esbmonde.htm). Consumption of BSE-infected food-stuffs has been shown to be a significant risk factor for the development of variant CJD (vCJD) in humans (Will et al., 2000; Foster et al., 2001a, b; Jeffrey et al., 2001; Hunter, 2003; Bellworthy et al., 2005a; Andréoletti et al., 2006; van Keulen et al., 2008). Therefore it is possible that, if BSE entered a sheep flock, lateral transmission may be more efficient than in cattle. To date, surveillance using biochemical tests that can discriminate between experimental ovine BSE and scrapie has failed to detect any evidence of naturally BSE-infected sheep (Thuring et al., 2004; Eloit et al., 2005; Sharpe et al., 2005; Stack et al., 2006), although there have been one confirmed case (Eloit et al., 2005) and one suspected case (Jeffrey et al., 2006) of natural BSE in goats. The risk to the human population of BSE in sheep requires further investigation and key to estimating such a risk is the calculation of the prevalence of BSE in sheep at any given point in time, which is in turn dependent on the susceptibility of sheep to the BSE agent and the efficiency of subsequent sheep-to-sheep and sheep-to-human passage (Baylis, 2002; Ferguson et al., 2002; Kao et al., 2002; Fryer et al., 2007). Inaccurate data on BSE-susceptible ovine genotypes could potentially have a

A supplementary table detailing the ovine PRNP ORF PCR amplification conditions used is available with the online version of this paper.
significant effect on the assessment of risk of BSE in sheep to the human consumer (Kao et al., 2003).

As with scrapie, sheep with different PRNP genotypes have been shown to differ in their susceptibility to BSE (Foster et al., 2001a, b; Jeffrey et al., 2001; Houston et al., 2003; Andrèoletti et al., 2006; Goldmann et al., 2006). The PRNP gene encodes the cellular prion protein (PrP\textsuperscript{C}), which can undergo misfolding to a disease-associated scrapie form (PrP\textsuperscript{Sc}) thought to be an integral component of the infectious prion agent (Prusiner, 1982). The ovine PRNP open reading frame (ORF) is 256 codons in length; three codons, 136, 154 and 171, are the most commonly observed variable sites in the gene and can affect resistance and susceptibility to classical scrapie in sheep. The dimorphic codon 136 can be either valine (V) or arginine (A), codon 154 can be arginine (R) or histidine (H) and the polymorphic codon 171 is most commonly glutamine (Q), arginine (R) or histidine (H). The three-codon allele is usually expressed as, for example, VRQ and the genotype as VRQ/ARQ. Additional PRNP mutations are most commonly seen in association with the ARQ allele, thought to be the ancestral wild-type allele (Goldmann et al., 2005).

ARQ/ARQ sheep are considered to be highly susceptible to BSE (Goldmann et al., 1994; Foster et al., 2001a; Houston et al., 2003; van Keulen et al., 2008) and are therefore used frequently in experimental challenges of BSE, such as those undertaken at the VLA (UK) and the Institut National de la Recherche Agronomique (INRA, France). However, in the course of several independent studies, small numbers of ARQ/ARQ sheep were observed to be resistant to challenge with BSE, either by displaying an extended survival period or failing to develop disease. We analysed samples and data from these studies further to ascertain whether genetics could explain the divergent results.

Suffolk sheep of the ARQ/ARQ genotype were sourced from the Defra New Zealand-derived flock (Simmons et al., 2009) and Romney sheep from a scrapie-free flock closed since 1972, and were used in a range of BSE-transmission studies at the VLA and INRA. Sheep received either a 5 g oral dose or a 0.1 g (VLA) or 0.05 g (INRA) intracerebral (IC) dose of BSE-infected bovine or ovine brainstem as described previously (Bellworthy et al., 2005a; Andrèoletti et al., 2006). Sheep were euthanized either at set time points or when they developed clinical disease. TSE diagnosis was carried out by immunohistochemistry, Western blotting and Bio-Rad Sheep and Goat ELISA on the brainstem (Gavier-Widén et al., 2005; Stack et al., 2006; Simmons et al., 2007).

The full ovine PRNP genotype was obtained by sequencing of the complete ORF of the PRNP gene as described previously (Saunders et al., 2006) for VLA samples and in Supplementary Table S1 (available in JGV Online) for INRA samples. For the purposes of this study, full PRNP ORF sequence data were obtained from BSE-dosed and control animals consisting of 97 Suffolk and 203 Romney breed ARQ/ARQ sheep housed at the VLA and 32 ARQ/ARQ INRA-based Suffolk sheep.

PRNP ORF sequencing revealed one additional polymorphism in the Suffolk-breed sheep and no PRNP polymorphisms in the Romney sheep. Suffolk animals were found to contain a mutation resulting in a methionine-to-threonine amino acid change at codon 112 (M112T), giving an M\textsuperscript{112}ARQ (wild-type, shortened to MARQ) to T\textsuperscript{112}ARQ (shortened to TARQ) variant.

In order to assess the effect of a particular PRNP allele on BSE susceptibility or resistance, a subset of BSE-dosed Suffolk sheep at VLA and INRA were selected from the various studies for further analysis. The animals selected included those inoculated experimentally with what could be considered an effective BSE dose (defined as \( \geq 5 \) g bovine or ovine BSE via the oral route or \( \geq 0.1 \) g bovine BSE or \( \geq 0.05 \) g ovine BSE via the IC route for the purposes of this analysis) and those culled after BSE became biochemically detectable (\( \geq 600 \) days post-inoculation (p.i.) for the orally challenged 5 g bovine BSE timed-cull group). Animals exposed to a low effective dose, culled before infection is detectable or exposed through 'natural' transmission (through contact with infected sheep or environment) are liable to result in a failure of transmitted infectivity in some animals due to factors that are independent of PRNP genetics. Therefore, data from 60 (35 MARQ/MARQ, 22 MARQ/TARQ and three TARQ/TARQ) of the 129 Suffolk animals genotyped, including some undosed control animals, were not utilized further.

The 69 Suffolk sheep selected and used to explore whether there is an association between PRNP genotype and BSE resistance and susceptibility are described in Table 1. Clinical disease was not observed in timed culls, end-of-experiment culls or intercurrent casualties.

All 47 BSE-positive Suffolk sheep carried the MARQ/MARQ PRNP genotype (Table 2). Of the 22 sheep that were BSE-negative or were still alive at the end of the study, 17 carried one TARQ allele and three carried two TARQ alleles (Table 2). Using Fisher's exact test, there is a highly statistically significant association between PRNP genotype and disease outcome in Suffolk sheep (\( P<0.0001 \)). Whilst the majority of resistant sheep carry at least one TARQ allele, there are two Suffolk sheep and numerous Romney sheep that exhibit BSE resistance in the absence of the TARQ allele. Therefore, some other, as-yet-unknown factor, potentially a non-coding region of the PRNP gene (Saunders et al., 2009), must be responsible in these cases.

Fourteen BSE-challenged sheep were still alive at the time of writing (March 2009), 715–3495 days p.i. (Table 1); 13 of these carry at least one TARQ allele. The mean survival time is longer than the mean incubation period for a comparable BSE challenge in a MARQ/MARQ susceptible animal (Fig. 1).

A dose–response model used previously for an oral attack rate study of BSE (Wells et al., 2007) was fitted to each
BSE-challenge group. In short, the model consists of a logistic-regression model for the probability of survival versus the log dose, but allowing for the possibility that the log-normal incubation period was longer than the time for which the animals have been observed. The model is fitted to the data by maximum likelihood and allows comparison of the impact of genotype on the dose–response via likelihood-ratio tests.

For sheep dosed orally with 5 g bovine BSE, a likelihood-ratio test showed that there was a statistically significant difference ($P<0.001$) between the dose–response of sheep

Table 1. Summary of 69 Suffolk sheep challenged with an ‘effective’ BSE dose used to assess the proportion of sheep carrying a particular $PRNP$ allele

Genotype shown is at $PRNP$ codons 112, 136, 154 and 171. Abbreviations: EoE, end-of-experiment cull; IC, intracerebral; PO, per os (oral); TC, timed cull.

<table>
<thead>
<tr>
<th>$PRNP$ genotype</th>
<th>$n^*$</th>
<th>Inoculum</th>
<th>Dose (g)</th>
<th>Route</th>
<th>Age at inoculation (weeks)</th>
<th>Reason for cull</th>
<th>Days p.i. (sd)</th>
<th>BSE diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VLA sheep</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>7</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>12</td>
<td>TC $\times$ 4</td>
<td>681–688</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clinical $\times$ 3</td>
<td>742–860 (66)</td>
<td></td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>1</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>12</td>
<td>–</td>
<td>3495</td>
<td>Alive</td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>2</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>28</td>
<td>EoE</td>
<td>2642, 2397</td>
<td>Negative</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>4</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>28</td>
<td>Clinical</td>
<td>570–800 (105)</td>
<td>Positive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>1</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>28</td>
<td>Casualty</td>
<td>1587</td>
<td>Negative</td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>2</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>26</td>
<td>–</td>
<td>2310</td>
<td>Alive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>1</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>26</td>
<td>–</td>
<td>2310</td>
<td>Alive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>10</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>26</td>
<td>Clinical</td>
<td>655–1470 (383)</td>
<td>Positive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>5</td>
<td>Bovine BSE</td>
<td>5</td>
<td>PO</td>
<td>30</td>
<td>Clinical</td>
<td>589–1048 (187)</td>
<td>Positive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>2</td>
<td>Bovine BSE</td>
<td>0.1</td>
<td>IC</td>
<td>35</td>
<td>Clinical</td>
<td>519, 566</td>
<td>Positive</td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>1</td>
<td>Bovine BSE</td>
<td>0.1</td>
<td>IC</td>
<td>22</td>
<td>–</td>
<td>880</td>
<td>Alive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>3</td>
<td>Bovine BSE</td>
<td>0.1</td>
<td>IC</td>
<td>22</td>
<td>Clinical</td>
<td>397–551 (89)</td>
<td>Positive</td>
</tr>
<tr>
<td>TARQ/TARQ</td>
<td>1</td>
<td>Bovine BSE</td>
<td>0.1</td>
<td>IC</td>
<td>22</td>
<td>–</td>
<td>880</td>
<td>Alive</td>
</tr>
<tr>
<td><strong>INRA sheep</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>4</td>
<td>Ovine BSE</td>
<td>5</td>
<td>PO</td>
<td>0–2</td>
<td>TC $\times$ 2</td>
<td>143, 144</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TC $\times$ 2</td>
<td>303, 313</td>
<td></td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>2</td>
<td>Ovine BSE</td>
<td>5</td>
<td>PO</td>
<td>0–2</td>
<td>TC</td>
<td>143, 304</td>
<td>Negative</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>2</td>
<td>Ovine BSE</td>
<td>5</td>
<td>PO</td>
<td>0–2</td>
<td>Clinical</td>
<td>575, 576</td>
<td>Positive</td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>7</td>
<td>Ovine BSE</td>
<td>5</td>
<td>PO</td>
<td>260</td>
<td>TC $\times$ 3</td>
<td>380–381 (0.6)</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TC $\times$ 4</td>
<td>602–604 (1)</td>
<td></td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>3</td>
<td>Ovine BSE</td>
<td>5</td>
<td>PO</td>
<td>260</td>
<td>TC $\times$ 2</td>
<td>381, 381</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TC $\times$ 1</td>
<td>602</td>
<td></td>
</tr>
<tr>
<td>MARQ/MARQ</td>
<td>3*</td>
<td>Ovine BSE</td>
<td>0.05</td>
<td>IC</td>
<td>39</td>
<td>Clinical</td>
<td>435–505 (35)</td>
<td>Positive</td>
</tr>
<tr>
<td>MARQ/TARQ</td>
<td>3*</td>
<td>Ovine BSE</td>
<td>0.05</td>
<td>IC</td>
<td>39–43</td>
<td>–</td>
<td>715–1094 (219)</td>
<td>Alive</td>
</tr>
<tr>
<td>TARQ/TARQ</td>
<td>3*</td>
<td>Ovine BSE</td>
<td>0.05</td>
<td>IC</td>
<td>91</td>
<td>–</td>
<td>715 (0)</td>
<td>Alive</td>
</tr>
</tbody>
</table>

*All sheep were randomly selected ARQ/ARQ sheep with the exception of those labelled with an asterisk, which were selected by $PRNP$ ORF genotype prior to dosing with BSE.

Table 2. Summary of the proportion of BSE-dosed sheep carrying a particular $PRNP$ genotype and associated with BSE susceptibility (BSE-positive) or resistance (alive/BSE-negative)

Data are given as no. sheep (percentage).

<table>
<thead>
<tr>
<th>Group</th>
<th>MARQ/MARQ</th>
<th>MARQ/TARQ</th>
<th>TARQ/TARQ</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSE-dosed</td>
<td>49 (100)</td>
<td>17 (100)</td>
<td>3 (100)</td>
<td>69 (100)</td>
</tr>
<tr>
<td>BSE-positive</td>
<td>47 (96)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>47 (68)</td>
</tr>
<tr>
<td>Alive/BSE-negative</td>
<td>2 (4)</td>
<td>17 (100)</td>
<td>3 (100)</td>
<td>22 (32)</td>
</tr>
</tbody>
</table>
with a MARQ/MARQ or a MARQ/TARQ genotype, with MARQ/TARQ animals having a low probability of being infected. The same result was obtained for combined 0.1 and 0.05 g IC ovine BSE-challenged animals, with non-MARQ/MARQ sheep having a low probability (P<0.001) of being infected. An almost significant difference (P=0.051) was obtained between MARQ/MARQ and MARQ/TARQ sheep in the IC bovine BSE-dosed group, with the MARQ/TARQ animals having a low probability of infection.

The M112T variant of the sheep PRNP gene has been reported previously in scrapie-free sheep (Ikeda et al., 1995; Heaton et al., 2003; Gombojav et al., 2004; Goldmann et al., 2005; Lan et al., 2006; Ohara et al., 2007; Serrano et al., 2007; Babar et al., 2009), sheep infected naturally with classical scrapie (Laplanche et al., 1993; Ikeda et al., 1995; Saunders et al., 2006; Vaccari et al., 2007), sheep challenged orally with scrapie (Laegreid et al., 2008) and in various breeds of sheep (Goldmann et al., 2005; Serrano et al., 2007; Babar et al., 2009). The Goldmann et al. (2005) study estimated that the TARQ allele accounts for 7% of ARQ alleles in 41 UK sheep flocks examined. The TARQ allele made up 18.6% of the ARQ alleles present in the Suffolk sheep from the VLA (36 of 194 alleles) and 15.2% of the randomly selected animals sequenced at INRA (seven of 46 alleles). Our findings may be biased by a founder effect in the New Zealand-derived flocks. However, a much higher prevalence of 31.6% TARQ alleles in Chinese Suffolk sheep has been reported (Zhang et al., 2004).

Scrapie resistance in orally challenged TARQ/TARQ sheep and extended survival times in MARQ/TARQ sheep have been reported previously (Laegreid et al., 2008). We have also observed an extended incubation period in MARQ/TARQ sheep (590 ± 41 days p.i.) compared with MARQ/MARQ sheep (372 ± 95 days p.i.) after IC inoculation with the Langlade scrapie strain (O. Andréoletti, unpublished data). Our study is the first to report the association between apparent BSE resistance and the ovine PRNP TARQ allele. However, it is possible that the MARQ/TARQ sheep are susceptible but asymptomatic, or are displaying an increased incubation period.

Laegreid et al. (2008) reported a median survival time of 1157 days in MARQ/TARQ sheep challenged orally with scrapie. Six of the MARQ/TARQ sheep challenged orally with bovine BSE in our study have survived beyond this period, with one animal still alive at 3495 days p.i. (Table 1). One of the six, culled at 2397 days p.i., displayed no clinical or diagnostic signs of BSE, suggesting that the TARQ-carrying resistant sheep are not asymptomatic carriers of the infection, as reported previously in sheep with apparently resistant genotypes (Ronzon et al., 2006). However, long incubation periods should not be interpreted as absolute resistance; therefore, confirmation by mouse bioassay and examination of a wider range of tissues is required to substantiate this finding. Furthermore, three of the six MARQ/TARQ sheep, alive at 3495, 2310 and 2310 days p.i., have survived beyond the longest incubation period of orally BSE-dosed VRQ/VRQ sheep (1825 days p.i.) (Bellworthy et al., 2008).

It is interesting to note that the highly effective IC route of challenge, which, with a dose of 0.1 g, shortens the incubation period of bovine and ovine BSE in MARQ/MARQ sheep compared with the incubation period of a 5 g oral dose (Fig. 1), does not overcome the transmission barrier of TARQ-carrying sheep challenged with bovine or ovine BSE in our study. The apparent resistance of ARR/ARR sheep to bovine BSE was first overcome by IC challenge, resulting in incubation periods of 1008–1127 days p.i. (Houston et al., 2003). These incubation periods are approximately equivalent to the current incubation periods of two bovine BSE IC-challenged (1490 days p.i.) and two ovine BSE IC-challenged (1094 days p.i.) MARQ/TARQ sheep still alive in our study at the time of writing. In vitro, the TARQ PrP<sup>C</sup> protein was found to convert poorly (Bossers et al., 2000) and recombinant TARQ PrP<sup>C</sup> proteins exhibited less β-sheet formation than MARQ PrP when exposed to copper (Yang et al., 2008), suggesting that MARQ PrP<sup>C</sup> may be converted more readily to pathogenic PrP<sup>Sc</sup> than TARQ PrP<sup>C</sup>. Although the number of animals within an individual BSE-challenge group is small, by combining data from several
studies, we have consistently found that the TARQ allele exhibits a protective effect on the transmission of BSE to sheep. Furthermore, animals carrying at least one TARQ allele have so far been resistant to ongoing challenges to assess ‘natural’ sheep-to-sheep BSE transmission after exposure to BSE-infected sheep at both the VLA (Bellworthy et al., 2005b) and INRA (F. Lantier, P. Berthon, I. Lantier & O. Andréoletti, unpublished data).

Another ovine PRNP ARQ allele with a proline-to-leucine variant at codon 168 (ARL168Q) has been reported to increase resistance to experimental intravenous inoculation with bovine BSE and exposure via blood transfusion from sheep infected with BSE (Goldmann et al., 2006). Our report emphasizes further the importance of obtaining the full ORF sequence of the ovine PRNP gene when studying the transmission of TSEs, and supports previous reports that PrP variants other than the commonly reported 136, 154 and 171 codons can modulate resistance and susceptibility to different TSE strains in sheep (Moum et al., 2005; Goldmann et al., 2006; Laegreid et al., 2008). Such information can inform risk assessments of BSE in sheep, breeding for resistance to scrapie and BSE in sheep, and the design and interpretation of experimental TSE-challenge data.

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References


