

Published online ahead of print on 11 August 2006 as DOI 10.1099/vir.0.81779-0

PrP genotypes of atypical scrapie cases in Great Britain

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Great Britain and elsewhere have detected atypical scrapie infection in sheep with PrP genotypes thought to be genetically resistant to the classical form of scrapie. DNA sequencing of the PrP gene of British atypical scrapie cases ($n=69$), classical scrapie cases ($n=59$) and scrapie-free controls ($n=138$) was undertaken to identify whether PrP variants, other than the three well-characterized polymorphic codons, influenced susceptibility to atypical scrapie infection. Four non-synonymous changes, M112T, M137T, L141F and P241S, were detected that are most probably associated with the A¹³⁶R¹⁵⁴Q¹⁷¹ haplotype. Only the PrP variant containing a phenylalanine residue at amino acid position 141 was found to be associated more commonly with the atypical scrapie cases. In addition to the single nucleotide polymorphisms associated with the ARQ allele, two out of nine atypical scrapie cases with the ARR/ARR genotype were found to contain a 24 bp insertion, leading to an additional octapeptide repeat. In terms of PrP genetics, one classification of the GB scrapie cases examined in this study would place animals carrying any homozygous or heterozygous combination of ARR, AHQ or AF¹⁴¹RQ alleles, or any one of these alleles when paired with ARQ, as being susceptible to atypical scrapie infection, and animals heterozygous or homozygous for VRQ and homozygous for ARQ as being susceptible to classical scrapie disease. The AHQ PrP allele was associated with the highest incidence of atypical scrapie (263 per 100 000 alleles), whilst VRQ was associated with the lowest incidence (10 per 100 000 alleles).

INTRODUCTION

Scrapie is a member of the transmissible spongiform encephalopathy (TSE) family of diseases, and its natural host is sheep and goats. Other TSE diseases include Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cattle. TSEs are degenerative disorders of the nervous system and are fatal. Whilst these diseases can be acquired, inherited or of idiopathic origin, the prion protein (PrP) always plays a central role (Prusiner, 1982). PrP can exist in a physiological conformation (PrP^C), the exact role of which is not fully understood, or in a TSE disease-associated isoform (PrP^{Sc}), which is thought to be the infectious agent. PrP^{Sc} has an increased resistance to digestion with proteases such as proteinase K (PK), and this biochemical characteristic is exploited in tests where the detection of the PK-resistant form of PrP is considered diagnostic for TSE disease.

The gene that encodes the ovine PrP has three exons, and the protein-coding region or open reading frame (ORF) of 768 bp is contained entirely within exon 3. DNA sequence analysis of the sheep PrP gene has so far found 26 polymorphic codons that result in an amino acid change (Goldmann *et al.*, 2005). Certain PrP polymorphisms are known to be associated with altered susceptibility to scrapie infection in sheep (Goldmann *et al.*, 1994). In the GB sheep population, the three ovine PrP polymorphic codons that are commonly linked to scrapie susceptibility are codons 136, 154 and 171 (three-codon genotype). Codon 136 encodes either alanine (A136) or valine (V136), codon 154 either arginine (R154) or histidine (H154), and codon 171, glutamine (Q171), arginine (R171) or histidine (H171). In particular, valine at residue 136 conveys scrapie susceptibility, whereas an alanine residue can result in relative resistance. At position 171, a glutamine residue conveys susceptibility, whereas arginine at this position results in an apparently resistant allele. The ancestral sheep allele is presumably A¹³⁶R¹⁵⁴Q¹⁷¹ (shortened to ARQ). This allele plus those generated through the substitution of one of its amino acids make up the five most common ovine PrP alleles, namely ARQ, VRQ, AHQ, ARR and ARH. Free permutation of these five alleles leads to 15 possible genotypes in the diploid organism, e.g. heterozygosity for VRQ and ARQ (VRQ/ARQ) or homozygosity for ARR (ARR/ARR).

The National Scrapie Plan (NSP) in the UK has focused the breeding of sheep to produce a higher prevalence of scrapie-resistant alleles in the national flock in an attempt to eradicate scrapie and the possibility of BSE in sheep (Dawson *et al.*, 1998). For this purpose, PrP genotypes have been typed to reflect their degree of resistance and susceptibility to the development of clinical scrapie. The type 1 genotype is ARR/ARR and is considered to be the most scrapie-resistant; resistance is reduced through to the type 5 group of genotypes, made up of the VRQ-containing genotypes AHQ/VRQ, ARH/VRQ, ARQ/VRQ and VRQ/VRQ, which are considered to be the most scrapie-susceptible. Type 4 consists of a single genotype that is heterozygous for the most susceptible and most resistant alleles and considered to be genetically susceptible to scrapie (ARR/VRQ). Type 2 genotypes, containing the remaining

ARR-heterozygous genotypes (ARR/AHQ, ARR/ARH and ARR/ARQ), are also considered resistant, but need careful selection when breeding; type 3 is the largest group and consists of all genotypes that do not carry the ARR or VRQ alleles (AHQ/AHQ, AHQ/ARH, AHQ/ARQ, ARH/ARH, ARH/ARQ and ARQ/ARQ).

In order to meet an EU Commission regulation (999/2001), a random sample of sheep over 18 months of age was targeted through active scrapie surveillance in Great Britain (GB). Between January 2002 and March 2003, an abattoir survey including the collection of samples from 50 630 non-clinical sheep was carried out; these samples were tested for scrapie and their three-codon PrP genotype was determined. Of these, 29 201 animals were tested for scrapie by using a standard immunoassay (Bio-Rad Platelia ELISA). Twenty-four of these animals tested positive for TSE by both ELISA and the confirmatory immunohistochemistry (IHC) diagnostic tests and were therefore considered to be classical scrapie cases. A further 28 tested positive by ELISA, but negative by IHC, making their TSE status unclassifiable (Wilesmith *et al.*, 2003). The abattoir survey continued from April 2003 to December 2003, during which time an additional 50 735 samples were screened for scrapie by using the ELISA test, including some fallen-stock sheep. A further 28 classical scrapie cases and 35 unconfirmed scrapie cases were identified; in this part of the survey, only the ELISA-positive samples were three-codon PrP-genotyped (Wilesmith *et al.*, 2004). All of the ELISA-positive scrapie cases that could not be confirmed by IHC were termed atypical scrapie and consisted of over half (54.8 %) of the total scrapie cases detected in this survey over this time period.

Improved IHC methodologies can now confirm asymptomatic atypical scrapie infection and distinguish it from the classical scrapie infection associated with clinical disease (Anonymous, 2005). The GB atypical scrapie cases have been examined further by biochemical and histological methods and these studies indicate a possibly novel form of prion or prion protein disorder (Anonymous, 2005; Everest *et al.*, 2006). This study focuses on the investigation of a possible correlation between PrP genetics and the newly emerging asymptomatic atypical scrapie cases in GB. Such an association has previously been reported for the atypical scrapie strain Nor98, which was shown to be associated with the AHQ allele and the ARQ allele carrying a phenylalanine residue at position 141 (Benestad *et al.*, 2003; Moum *et al.*, 2005). The particular aim of this study was to identify whether PrP variants, other than the common polymorphic codons at positions 136, 154 and 171, influence susceptibility to atypical scrapie infection.

METHODS

Samples. This study examined three sample groups based on their scrapie status: 1, atypical scrapie cases ($n=69$), so classified due to their original IHC-negative and Bio-Rad Platelia-positive diagnosis; 2, classical scrapie cases ($n=59$), which were IHC-positive and Bio-Rad Platelia-positive; 3, scrapie-negative cases ($n=138$), as determined by IHC- and Bio-Rad (TeSeE sheep and goat)-negative diagnoses. The atypical scrapie and classical scrapie groups consisted of all such abattoir-surveillance cases detected over the 2 year period described previously, plus fallen-stock survey cases (eight classical and seven atypical scrapie cases) detected from April to December 2003, where samples were available (Table 1). Classical scrapie cases positive by Western blot, but not tested by the Bio-Rad ELISA ($n=39$), were not included for sequencing, as it is not known whether both tests detect PrP^{res} from all PrP variants with equal sensitivity and specificity. The negative-control sample set was chosen from the abattoir-survey animals based on their PrP 136, 154 and 171 genotype and therefore does not represent a case-control study group. The scrapie-negative cases were selected to provide a number equal to that found in the combined atypical and classical scrapie groups, with a minimum of five of each genotype. This was achieved for all of the genotypes with the exception of ARH/VRQ and ARQ/ARQ. The possibility of matching controls on criteria other than genotype was considered, but would have proven difficult. The sheep breeds and age of animals in the study are unknown and, as the farm location from which the sheep originated was not available, this information could not be traced. The majority of abattoir animals in all three groups were thought to be older than 18 months of age and in apparently good health that passed through 44 British abattoirs; these abattoirs slaughter 93 % of sheep in GB (Wilesmith *et al.*, 2003). The 15 fallen-stock cases were sheep found on farms or that died in transit to the abattoir and were therefore not of sound health.

DNA extraction. DNA for full ORF sequencing was extracted from 25 mg brain residual medulla tissue by using a DNeasy 96 Tissue kit (Qiagen) according to the manufacturer's instructions, with a final volume of 150 μ l.

Full sequencing of a 1.1 kbp fragment including the PrP ORF. A guide sequence, available from GenBank, from a USA Suffolk sheep (accession no. U67922; Lee *et al.*, 1998) was used to assist with primer design. The 1.1 kbp region of interest, including the entire PrP ORF, ranges from bp 22218 to 23318 of the guide sequence and covers from -60 bp from the PrP atg start codon to +1040 (stop codon at +770). With a view to the possibility of locating novel DNA polymorphisms, the area of interest was amplified with two subtly different primer pairs. This precaution aimed to avoid any allelic dropout that could be caused by a novel mutation in a primer sequence and thereby lead to the preferential amplification of only one of the two alleles present. Primer pairs used were F1 (5'-CATTTATGACCTAGAATGTTTATAGCTGATGCCA-3')

and R1 (5'-TTGAATGAATATTATGTGGCCTCCTTCCAGAC-3'), equating to bp 22150–23378 of the guide sequence, and F2 (5'-ATTTATGACCTAGAATGTTTATAGCTGATGCCACT-3') and R2 (5'-CCAGTTTTGTTTTTTTTGAATGAATATTATGTGGC-3'), giving a product equivalent to bp 22151–23392 of the guide sequence. The amplification was conducted in a 10 μ l final reaction volume containing 1 \times Qiagen HotstarTaq buffer, 2 mM MgCl₂, 200 μ M dNTPs, 100 μ g BSA ml⁻¹, 0.05 U Qiagen HotstarTaq polymerase μ l⁻¹, 200 nM PCR primers and 5 μ l extracted genomic DNA sample. Thermal cycling was undertaken by using an initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 66 °C for 60 s and 72 °C for 60 s. Both strands of both PCR products were sequenced by using three forward and three reverse primers approximately 300–400 bp apart: 30F, 5'-ATGACCTAGAATGTTTATAGCTGATGCCA CTGC-3'; 344F, 5'-CATGGTGGTGGAGGCTGGGGTC-3'; 680F, 5'-GGGAGAACTTCACCGAA ACTGACATCA-3'; 375R, 5'-GCTTCATGTTGGTTTTTGGCTTACTGG-3'; 710R, 5'-GGATTCTC TCTGGTACTGGGTGATGCA-3'; 1131R, 5'-TTGAATGAATATTATGTGGCCTCCTTCCAGA-3'. Sequencing was performed by using BigDye Terminator v3.1 reagents (Applied Biosystems). Cycle-sequencing reactions were undertaken by using thermal-cycler conditions of an initial denaturation at 96 °C for 60 s, followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Prior to loading on an ABI 3100 genetic analyser, the sequencing product was purified by using a CleanSEQ kit (Agencourt). The sequence data obtained were compared with the ORF of a Suffolk sheep (GenBank accession no. U67922; Lee *et al.*, 1998) by using SeqScape software v2.5 (Applied Biosystems) to identify DNA polymorphisms. PCR amplifications and DNA sequencing were undertaken by Orchid Cellmark (Abingdon, Oxon, UK).

RESULTS

Full PrP gene sequence was obtained for all of the 266 samples examined and the 136, 154 and 171 PrP genotype reported previously for the abattoir survey by using a different sequencing approach (Everest *et al.*, 2006) was always confirmed, providing evidence that allelic dropout had not occurred. Furthermore, for each sample, the ORF sequence generated from the two independently amplified 1·1 kbp PCR fragments was identical, making it unlikely that, for homozygous samples, allelic dropout had occurred. In addition to the 136, 154 and 171 common variants, full DNA sequencing of the ORF revealed two synonymous polymorphisms, four non-synonymous changes and a novel 24 bp insertion. The synonymous changes were at bp 691 and 711 (numbered from the a of the atg start codon) in codons 231 and 237, respectively; the non-synonymous changes were at codon positions 112 (M112T), 137 (M137T), 141 (L141F) and 241 (P241S) in a subset of animals (Table 2). All of these codon changes have been reported previously (reviewed by Goldmann *et al.*, 2005).

Two of these polymorphisms were rare; the M137T change was found in only one scrapie-negative animal. The M112T change was found in three cases of classical scrapie; any possible significance of an M112T association with classical scrapie is not possible to define, as the numbers are too small. The other two codon changes were more frequent; the P241S variant occurred in four atypical cases, four classical scrapie cases and 11 scrapie-negative controls. Overall, the L141F codon change was the most commonly observed; however, only four F141L codon changes were associated with classical scrapie (6·8 % of these cases), compared with 20 cases (29·0 %) of atypical scrapie (Table 2). This variant was also seen in eight scrapie-negative sheep (5·8 %). The majority (83 %) of the L141F codon changes detected in the total scrapie cases (atypical plus classical) are associated with atypical scrapie. Therefore, whereas the L141F codon change is represented equally in classical scrapie and the negative subset, it is clearly over-represented in atypical scrapie cases. Three of the other common alleles (AHQ, ARH and VRQ) did not carry any additional PrP polymorphisms.

In addition to single nucleotide polymorphisms, two atypical scrapie cases with the ARR/ARR genotype were found to contain a 24 bp insertion, leading to an additional octapeptide repeat; one animal was homozygous for the six-octapeptide repeat variant, whilst the second was heterozygous (Table 2).

Two previously reported synonymous polymorphisms at bp 691 and 711 in codons 231 and 237 (Heaton *et al.*, 2003) were always linked and always heterozygous (691 a/a to a/c; 711 c/c to c/g). The silent mutations were found in all study groups. The paired silent mutations were found to be associated with all genotypes containing an ARH allele (14/14), as reported by Slate (2005), and were also associated with some genotypes containing at least one ARQ or AHQ allele. Although the haplotype containing these silent mutations is not proven, they do not seem to be associated with the ARR allele (0/19 ARR/ARR animals) and this may therefore account

for their reduced presence in the atypical scrapie cases (5.8 %, compared with 11.5 % of classical scrapie cases and 15.9 % of selected negative cases).

The additional coding polymorphisms were stratified according to the three-codon genotype (Table 3) to give the full PrP genotype. The rare polymorphisms M112T, M137T, F141L and the P241S mutation are most likely to be in association with the ARQ allele, as animals heterozygous for these codon variants were always associated with at least one ARQ allele. In the case of the P241S polymorphism, a single scrapie-negative case, homozygous for serine at codon 241, was associated with a homozygous ARQ genotype, confirming the ARQS²⁴¹ haplotype. Occurring more frequently, the F141L variant can be assigned more confidently to the ARQ haplotype. Due to the absence of the F141L codon change in any of the AHQ-, ARR- or VRQ-homozygous animals ($n=66$) and the six homozygous F141L cases being found in ARQ/ARQ animals, the evidence in this study further supports the finding that AF¹⁴¹RQ forms a haplotype, as reported by Moum *et al.* (2005) and references therein. The novel six-octapeptide repeat variant was clearly in association with the ARR haplotype, as both cases where this insertion was observed were ARR-homozygous animals.

Interestingly, with the exception of one animal (AF¹⁴¹RQ/VRQ), atypical cases did not contain the valine residue at codon 136 and are therefore found collectively in the more resistant genotypes of types 1, 2 and 3 (Dawson *et al.*, 1998) for the purposes of the NSP (Wilesmith *et al.*, 2004). As expected, the genotypes associated most closely with classical scrapie include ARQ/VRQ, VRQ/VRQ, ARR/VRQ and ARH/VRQ, which are NSP type 4 or 5, or those considered to be genetically susceptible to scrapie. Based on only the three-codon genotype, the ARQ/ARQ genotype was distributed most evenly, with 10.1 % of the atypical scrapie cases and 13.1 % of the classical scrapie cases having this type 3 NSP genotype (Table 1). The presence or absence of the L141F codon change in ARQ/ARQ animals now clearly separates this group with respect to scrapie status, with the ARQ/AF¹⁴¹RQ (1/1) and AF¹⁴¹RQ/AF¹⁴¹RQ (6/6) genotypes noticeably associated with atypical scrapie and the ARQ/ARQ (6/6) genotype associated with classical scrapie (Table 3).

As seen in Table 3, only three genotypes present incidence of both atypical and classical scrapie (AHQ/AHQ, AHQ/AF¹⁴¹RQ and VRQ/AF¹⁴¹RQ). Notably, two of these genotypes contain the AHQ allele (AHQ/AHQ and AHQ/AF¹⁴¹RQ) and these are associated more commonly with atypical scrapie (14.5 and 7.2 % of all cases, respectively) than in classical scrapie (1.7 and 1.7 % of all cases, respectively). The VRQ/AF¹⁴¹RQ genotype is seen more frequently in classical scrapie (5.1 % of cases) than atypical scrapie (1.4 % of cases). The *P* value in the final column of Table 3 compares the proportions of atypical and classical scrapie cases by genotype, using genotypes made up of the six most common alleles only. An additional Fisher's exact test found a highly significant difference ($P<0.001$) in the overall distributions of genotypes between atypical and classical scrapie cases.

The three most common atypical scrapie PrP alleles are ARR, AHQ and AF¹⁴¹RQ, collectively making up 87 % of the alleles present in this group, whereas the three most

common PrP alleles in the classical scrapie cases are VRQ, ARQ and ARR, making up 92 % (Table 4). Moreover, all 69 (100 %) of the atypical scrapie sheep carry at least one of these 'high-frequency' atypical scrapie alleles (ARR, AHQ or AF¹⁴¹RQ), and 52 (75.3 %) carry two (Table 3). This compares with 20 (33.9 %) classical scrapie cases carrying at least one of these alleles and two (3.3 %) carrying two, leaving 39 (66.1 %) classical scrapie cases with genotypes that are lacking ARR, AHQ or AF¹⁴¹RQ alleles. In scrapie cases where only one of these 'high-frequency' atypical scrapie alleles is present (e.g. an ARR/VRQ or AHQ/ARQ genotype), the distribution of the second allele also differs between the atypical and classical scrapie cases. In the atypical scrapie cases, the most common second allele is wild-type ARQ (88.2 %), whereas in the classical scrapie cases, it is VRQ (100 %), suggesting that the VRQ allele may have a dominant effect over the three alleles associated most commonly with atypical scrapie, rendering VRQ-containing sheep more susceptible to classical scrapie.

In order to assess scrapie frequency with respect to PrP genetics, Table 5 shows the scrapie incidence by allele of all the ELISA-tested sheep in the abattoir and fallen-stock surveys from January 2002 to December 2003. The three-codon-genotyped abattoir-survey scrapie-negative data (January 2002 to March 2003), which, as a large, random sample, offers a good representation of the genotypic and allelic distribution in the national flock (Table 1, column 3), could not be used directly to calculate atypical scrapie incidence, as approximately 21 000 of the 48 000 animals had been tested by Western blot followed by IHC, which would not detect atypical scrapie cases. The allelic frequency observed in this sample set was therefore applied to the sample numbers that had been ELISA-tested in order to calculate scrapie incidence. Furthermore, scrapie incidence based on genotype was not calculated, as the frequency of genotypes including the AF¹⁴¹RQ allele is not available for the abattoir negative sample set. Incidence of atypical scrapie is associated most commonly with the AHQ allele (263 per 100 000 alleles) and least commonly with the VRQ allele (10 per 100 000 alleles), whereas classical scrapie is associated most commonly with the VRQ allele (552 per 100 000 alleles) and least commonly with the ARR and AHQ alleles (21 and 15 per 100 000 alleles, respectively). When the incidence of atypical and classical scrapie is combined, VRQ is the most commonly occurring allele (562 per 100 000 alleles) and ARR occurs the least (80 per 100 000 alleles). The AF¹⁴¹RQ allele is more than six times more likely to be associated with an atypical scrapie case than a classical scrapie case (195 vs 30 per 100 000 alleles) and also more than six times more likely to be associated with an atypical scrapie case than the wild-type ARQ allele (195 vs 31 per 100 000 alleles).

DISCUSSION

An active scrapie-surveillance programme that targeted sheep passing through British abattoirs found that the scrapie status of some animals was deemed atypical, as they could not be classified as truly scrapie-positive or -negative by using ELISA and contemporary IHC methodologies. A new type of ovine prion or prion protein disorder in GB was therefore identified, referred to here as atypical scrapie infection, and has been described in terms of its biochemical properties and PrP^{Sc} distribution (Anonymous, 2005; Everest *et al.*, 2006). In this study, the entire ORF of the PrP gene from 69 atypical scrapie and 59 classical scrapie cases and genotype-matched controls was sequenced. Results confirmed the association between atypical scrapie cases and PrP genotypes thought of as being scrapie-resistant. As reported previously, there is a strong bias in the GB atypical cases towards the ARR/ARR, ARR/AHQ, ARR/ARQ, AHQ/AHQ and AHQ/ARQ genotypes compared with the classical scrapie cases (Wilesmith *et al.*, 2003, 2004). The distribution of these genotypes was 13.0, 21.7, 13.0, 14.5 and 24.6 % (total 86.8 %), respectively, in the atypical cases, compared with 0.0, 0.0, 0.0, 1.7 and 1.7 % (total 3.4 %), respectively, in the classical scrapie cases (Tables 1 and 3). This study identified a novel variant of the ARR allele that leads to the insertion of 8 aa in the N-terminal octapeptide repeat region of the PrP protein, resulting in six tandem repeats instead of the usual ovine wild-type five octapeptide repeats. The two animals carrying this variant were both atypical scrapie cases, but with such small numbers that the significance of this cannot be determined. A further seven ARR-homozygous atypical scrapie cases contained no additional polymorphisms, thereby confirming that the additional octapeptide repeat is not in itself a requirement to render ARR/ARR sheep susceptible to atypical scrapie. Although codon polymorphisms at positions 112, 137 and 241 may not alter the susceptibility of the ARQ allele to atypical scrapie, this is not the case for the 141 polymorphic codon. Six cases of AF¹⁴¹RQ/AF¹⁴¹RQ sheep were diagnosed with atypical scrapie and none with classical scrapie, whereas six cases of ARQ/ARQ were diagnosed with classical scrapie and none with atypical scrapie. The AF¹⁴¹RQ allele makes up 19 % of the atypical scrapie alleles and only 3 % of the classical scrapie alleles, and is estimated to form 8 % of the alleles that make up the national flock (Table 4). The atypical scrapie incidence of the AF¹⁴¹RQ allele is 195 per 100 000 alleles, compared with 31 per 100 000 for ARQ alleles not containing this polymorphic codon. In contrast, the classical scrapie incidence of the AF¹⁴¹RQ allele is 30 per 100 000 alleles, compared with 77 per 100 000 for ARQ alleles with leucine at codon 141 (Table 5). Clearly, the PrP variant containing a phenylalanine residue at amino acid position 141 is associated more commonly with the atypical scrapie cases.

In terms of PrP genetics, a simple classification of the GB atypical cases examined in this study would place animals carrying any homozygous or heterozygous combination of ARR, AHQ or AF¹⁴¹RQ alleles or one of these alleles when paired with ARQ as being susceptible to atypical scrapie infection, and animals heterozygous or homozygous for VRQ and homozygous

for ARQ as being susceptible to classical scrapie. Out of the 128 cases (atypical and classical scrapie), two (1.6 %) classical scrapie cases and one (0.8 %) atypical scrapie case do not fit this basic categorization.

The genotypes that present cases of both atypical and classical scrapie (AHQ/AHQ, AHQ/AF¹⁴¹RQ and VRQ/AF¹⁴¹RQ) may represent a genuine broad susceptibility to different types of scrapie; certainly, this suggests that disease phenotype is not determined by PrP genotype alone.

A small number of PrP genotypes did not present atypical or classical scrapie, but were found in the abattoir scrapie-negative samples, namely AHQ/ARH (147), ARH/ARH (177), ARQ/ARH (529) and AHQ/VRQ (833), which collectively make up 3.4 % of the survey negative genotypes; it is of interest to note that three of the genotypes share an ARH allele. The least common ARH-containing genotype from the abattoir survey, ARH/VRQ (0.2 % of the scrapie-negative animals), did however present three classical scrapie cases, whilst ARH/ARR (1.4 % of the scrapie-negative animals) presented one atypical case. The ARH/VRQ genotype does have the second-greatest scrapie (classical) risk of UK sheep after VRQ/VRQ (Baylis *et al.*, 2004), so it is perhaps not surprising to see it in the classical scrapie group. The ARH allele in the GB atypical and classical scrapie cases was only found when paired with a dominant allele (VRQ or ARR) that could convey susceptibility to classical or atypical scrapie, respectively. The absence of AHQ/ARH, ARQ/ARH and particularly ARH/ARH genotypes from the scrapie groups in this study is most probably due to the relatively low number of these animals passing through British abattoirs (ARH accounts for only 1.86 % of the alleles present; Table 4).

GB is not alone in detecting such atypical scrapie cases associated with genotypes thought to be genetically resistant to the classical type of scrapie. Several factors may have enhanced the discovery of atypical scrapie cases, including the use of rapid diagnostic tests that appear to be more sensitive to the detection of PrP^{res} and the examination of pre- or non-clinical animals, as in the abattoir survey.

A new strain of scrapie, Nor98, first reported in five AHQ-homozygous and two AHQ/ARQ sheep in Norway, was found to have an unusual PrP^{Sc} distribution and glycoform (Benestad *et al.*, 2003). The authors suggested that detection of this Nor98 strain could have been previously overlooked in surveillance programmes. The genotype of the Nor98 cases is of interest, as these two genotypes make up 38 % of the GB atypical cases. A further report from Norway (Moum *et al.*, 2005) found that 38 Nor98 cases were associated strongly with AF¹⁴¹RQ and AHQ alleles, but, in contrast to the GB atypicals, not with the ARR allele, despite it being present in 25.6 % of Nor98 flock-mates (FMs). Nor98 was therefore not found in any ARR/ARR-genotype animals. No VRQ alleles were associated with Nor98, although they were well represented in the Nor98 FMs (12.5 %); therefore, this allele could confer partial or complete resistance to scrapie Nor98. A comparison of the allelic distribution of Nor98 (Moum *et al.*, 2005) and the GB atypical scrapie cases can be seen in Table 4. Genotypes identified with the

GB atypical cases, but not found in Nor98 scrapie, include ARH/ARR (1.5 % of Nor98 FMs), ARQ/ARR (15.9 % FMs), AF¹⁴¹RQ/VRQ (1.9 % FMs) and ARR/ARR (8.8 % FMs).

Until recently, there was little evidence of TSEs in the most resistant sheep genotype, ARR/ARR (Ikeda *et al.*, 1995); however, intracerebral inoculation of sheep of this genotype with BSE has caused disease (Houston *et al.*, 2003). There have also been several European reports of naturally occurring atypical scrapie in sheep with this scrapie-resistant genotype, but numbers are limited to two in Germany (Buschmann *et al.*, 2004a) and one in Portugal (Orge *et al.*, 2004), all detected through active surveillance by using rapid tests. This study is therefore exceptional in reporting atypical scrapie in nine ARR-homozygous sheep, two of which contained a novel additional octapeptide repeat not reported previously.

Further atypical cases have now been reported in several other European countries, including Germany, France, Belgium, Sweden, Ireland and Portugal (Buschmann *et al.*, 2004a, b; De Bosschere *et al.*, 2004; Gavier-Widen *et al.*, 2004; Lühken *et al.*, 2004; Madec *et al.*, 2004; Onnasch *et al.*, 2004; Orge *et al.*, 2004). The majority genotypes reported are AHQ/AHQ, ARR/AHQ, AHQ/ARQ, ARR/ARQ and ARQ/ARQ. The AHQ allele is most prominent in GB, Nor98 (Benestad *et al.*, 2003; Moum *et al.*, 2005) and in German Merinoland (Lühken *et al.*, 2004) atypical scrapie sheep. Other than the Norwegian studies, none of these reports have evidence of an AF¹⁴¹RQ allele association with atypical scrapie, which in some cases could be due to the limitations of the genotyping methods used; therefore, this association remains limited to Nor98 (Moum *et al.*, 2005) and the GB atypical cases in this study (Table 4).

The fact that the genotype distribution of the GB atypical scrapie cases does not correlate directly with results from other studies could suggest that the GB atypical cases represent more than one scrapie strain, possibly a Nor98-like strain and a further ARR susceptible strain. The biochemical study of the GB atypical cases (Everest *et al.*, 2006) does not observe the 12kDa PrP^{res} Western blot band associated consistently with Nor98 (Moum *et al.*, 2005) – in particular, it was not evident in the ARR/ARR GB atypical scrapie cases examined; however, neither was it consistently present in atypical scrapie cases of the AHQ/AHQ genotype.

The observation that NSP type 1 and 2 genotypes have been found to be associated closely with atypical scrapie cases (49 %) has raised concerns that should NSP type 1 and 2 sheep be sufficiently susceptible to BSE or atypical scrapie via natural transmission, the NSP might fail (Baylis & McIntyre, 2004). The NSP, launched in 2001, has effectively encouraged the proliferation of the scrapie-resistant ARR PrP haplotype, whilst selected breeding has restricted the proliferation of the susceptible VRQ haplotype. The result of the plan is reflected in the current allelic distribution of the national flock, where the ARR allele contributes 43 % and the VRQ just 6 % of alleles present. In terms of the incidence of total scrapie (atypical plus classical) as estimated with the data available here, the ARR and VRQ alleles have the lowest (80) and highest (562) incidence per 100 000 alleles, respectively, in ELISA-tested samples over a 2 year period (Table 5). Indeed, it should be noted that VRQ/VRQ scrapie-positive

animals could be under-represented in the abattoir survey, as although the sheep targeted were over 18 months of age, few sheep are slaughtered in GB between 18 and 36 months of age (Wilesmith *et al.*, 2003); therefore, many of the sheep could be older than 3 years of age. With the mean age at death from scrapie for VRQ-homozygous sheep being 3.2 years (Baylis & Goldmann, 2004), an unknown proportion of such scrapie-infected sheep would not have survived to an age to be included in the survey. In contrast, the high percentage (25.4 %) of ARR/VRQ sheep in the classical scrapie group is probably due to an extended incubation period in sheep of this genotype, estimated to be 5.6 years (Wilesmith *et al.*, 2004), suggesting that classical scrapie in sheep of this genotype may be largely undetected by passive surveillance that relies on clinical symptoms. Depending on the outcome of further investigations of current and future atypical scrapie cases in GB and elsewhere to monitor and characterize atypical scrapie cases, including incidence levels, strain identification, infectivity, risk to public health, disease phenotype and genotypic susceptibility, a review of the NSP may become appropriate. Indeed, there could be some argument for maintaining a broad range of PrP variants within the national flock in order to maintain resistance to possible variety of past, present and future TSE strains.

ACKNOWLEDGEMENTS

This work was supported by a grant from Defra, UK. The authors thank Paula Keyes and Steve Hawkins and their teams at VLA for sourcing and providing sample material, CERA, VLA, for additional information on the surveys and statistical analyses by Robin Sayers. They are also grateful to Hugh Spotswood and Brian McKeown from Orchid Cellmark for DNA sequencing and Wilfred Goldmann and Nora Hunter for useful discussions.

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Table 1. PrP genotype distribution (codons 136, 154 and 171) of scrapie-infected abattoir-survey and fallen-stock sheep used in this study (columns 1–2)

For comparison, the final group (column 3) shows the genotype distribution of the scrapie-negative sheep in the abattoir survey (January 2002 to March 2003) (Wilesmith *et al.*, 2003, 2004). This group also contained a relatively small number of genotypes not detected in groups 1 or 2 of this study; see Discussion for more details.

NSP type/genotype	Study groups used for sequencing of PrP ORF				3. Scrapie-free survey-negative (IHC ⁻ , ELISA ⁻)	
	1. Atypical scrapie (IHC ⁻ , ELISA ⁺)		2. Classical scrapie (IHC ⁺ , ELISA ⁻)		n	%
	n	%	n	%		
1:						
ARR/ARR	9	13.0	0	0.0	9 524	19.6
2:						
ARR/AHQ	15	21.7	0	0.0	4 563	9.4
ARR/ARH	1	1.4	0	0.0	694	1.4
ARR/ARQ	9	13.0	0	0.0	15 153	31.1
3:						
AHQ/AHQ	10	14.5	1	1.7	849	1.7
AHQ/ARQ	17	24.6	1	1.7	4 259	8.8
ARQ/ARQ	7	10.1	8	13.6	6 955	14.3
4:						
ARR/VRQ	0	0.0	15	25.4	2 660	5.5
5:						
ARH/VRQ	0	0.0	3	5.1	88	0.2
ARQ/VRQ	1	1.4	24	40.6	2 044	4.2
VRQ/VRQ	0	0.0	7	11.9	183	0.4
Total	69	100	59	100	46 972	96.5

Table 2. Additional PrP protein codon changes detected

Codon	Atypical (n=69)		Classical (n=59)		Negative (n=138)	
	n	%	n	%	n	%
M112T heterozygous	–	–	3	5.1	–	–
M137T heterozygous	–	–	–	–	1	0.7
L141F heterozygous	14	20.3	4	6.8	7	5.1
L141F homozygous	6	8.7	–	–	1	0.7
P241S heterozygous	4	5.8	4	6.8	10	7.2
P241S homozygous	–	–	–	–	1	0.7
6/6 octapeptide repeat	1	1.4	–	–	–	–
6/5 octapeptide repeat	1	1.4	–	–	–	–

Table 3. Full PrP genotype determined by sequencing (additional codon changes are shown in bold)

NSP type	Genotype	Full PrP ORF genotype (where different)	Scrapie-negative		Atypical scrapie		Classical scrapie		P value
			n	%	n	%	n	%	
1	}	ARR/ARR	10	7.3	7	10.1	–	–	{ 0.004
		ARR/ARR	–	–	1	1.4	–	–	
		ARR/ARR	–	–	1	1.4	–	–	
2	}	ARR/AHQ	17	12.3	15	21.7	–	–	<0.001
		ARR/ARH	6	4.4	1	1.4	–	–	1.000
		ARR/ARQ	4	2.9	2	2.9	–	–	{ 0.499
		ARR/ARQ	3	2.2	–	–	–	–	
		ARR/ARQ	2	1.4	7	10.1	–	–	0.015
		ARR/ARQ	2	1.4	7	10.1	–	–	0.015
3	}	AHQ/AHQ	11	8.0	10	14.5	1	1.7	0.011
		AHQ/ARQ	13	9.4	8	11.6	–	–	{ <0.001
		AHQ/ARQ	3	2.2	4	5.8	–	–	
		AHQ/ARQ	2	1.4	5	7.2	1	1.7	0.122
		ARQ/ARQ	8	5.8	–	–	6	10.2	{ 0.002
		ARQ/ARQ	2	1.4	–	–	2	3.4	
		ARQ/ARQ	1	0.7	–	–	–	–	0.030
ARQ/ARQ	1	0.7	6	8.7	–	–	1.000		
4	}	ARR/VRQ	16	11.6	–	–	15	25.4	<0.001
		ARH/VRQ	4	2.9	–	–	3	5.1	0.095
5	}	ARQ/VRQ	21	15.2	–	–	16	27.1	{ <0.001
		ARQ/VRQ	–	–	–	–	3	5.1	
		ARQ/VRQ	1	0.7	–	–	–	–	{ 0.334
		ARQ/VRQ	2	1.4	–	–	2	3.4	
		ARQ/VRQ	2	1.4	1	1.4	3	5.1	0.004
		VRQ/VRQ	8	5.8	–	–	7	11.8	0.004
Total			138	100	69	100	59	100	

*No. octapeptide repeats. In heterozygous animals, codon changes assigned to the ARQ haplotype are inferred. By using Fisher's exact test, the P value in the final column compares the proportions of atypical and classical scrapie cases of a single genotype (using genotypes consisting of the six most common alleles only, namely ARQ, AF¹⁴¹RQ, ARH, AHQ, ARR and VRQ), e.g. the first row of the last column compares 9/69 atypical vs 0/59 classical scrapie cases with the ARR/ARR genotype.

Table 4. Allelic distribution of atypical and classical scrapie cases

Allelic distribution of the atypical and classical scrapie cases in this study compared with the total scrapie-negative cases from the abattoir survey (Wilesmith *et al.*, 2003, 2004) and Nor98 scrapie cases (Moum *et al.*, 2005).

Allele	Survey scrapie-negative		Classical scrapie		Atypical scrapie		Nor98	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
AHQ	11 500	11.8	3	2.5	52	37.7	32	42.1
ARH	1 812	1.9	3	2.5	1	0.7	1	1.3
ARQ	28 142*	28.9	37	31.4	15	10.9	9	11.8
AF ¹⁴¹ RQ	7 753*	8.0	4	3.4	26	18.8	25	32.9
ARR	42 118	43.3	15	12.7	43	31.2	9	11.8
VRQ	5 991	6.2	57	48.3	1	0.7	0	0.0
Total	97 316	100.0	118	100.0	138	100.0	76	100.0

*These figures are estimated by using the observation of Goldmann *et al.* (2005) that 21.6 % of ARQ haplotypes carry the L141F codon change in 41 scrapie-infected and scrapie-free flocks in the UK.

Table 5. Scrapie incidence by allele

Allelic frequency, obtained from the genotyping of scrapie-negative sheep (January 2002 to March 2003, $n=48\ 658$) (29 201 tested by ELISA and 21 429 tested by Western blot), was used to calculate the alleles found in all Bio-Rad Platelia-negative animals (no. abattoir animals tested January 2002 to March 2003, 29 201; April 2003 to December 2003, 50 735; fallen stock, April 2003 to December 2003, 3400). From this, the scrapie incidence per 100 000 alleles was calculated.

Allele	Abattoir survey scrapie-negative (ELISA and Western blot)		ELISA scrapie-negative samples		Atypical scrapie		Classical scrapie		Total scrapie	
	<i>n</i>	Allelic frequency (%)	Calculated <i>n</i>	Calculated allelic frequency (%)	<i>n</i>	Incidence per 100 000 alleles	<i>n</i>	Incidence per 100 000 alleles	<i>n</i>	Incidence per 100 000 alleles
ARR	42 118	43.3	72 135	43.3	43	60	15	21	58	80
AHQ	11 500	11.8	19 696	11.8	52	263	3	15	55	279
AF ¹⁴¹ RQ	7 753*	8.0	13 278	8.0	26	195	4	30	30	225
ARH	1 812	1.9	3 103	1.9	1	32	3	97	4	129
ARQ	28 142*	28.9	48 198	28.9	15	31	37	77	52	108
VRQ	5 991	6.2	10 261	6.2	1	10	57	552	58	562
Total	97 316	100.0	166 672	100.0	138	83	118	71	257	154

*These figures are estimated by using the observation of Goldmann *et al.* (2005) as in Table 4. n.b. Four atypical and five classical scrapie cases from the survey were not analysed in this study, due to the absence of biological material.