Infectious titres of sheep scrapie and bovine spongiform encephalopathy agents cannot be accurately predicted from quantitative laboratory test results

Lorenzo González, Leigh Thorne, Martin Jeffrey, Stuart Martin, John Spiropoulos, Katy E. Beck, Richard W. Lockey, Christopher M. Vickery, Thomas Holder and Linda Terry

Animal Health and Veterinary Laboratories Agency (AHVLA), AHVLA-Lasswade, Pentlands Science Park, Penicuick, Midlothian EH26 0PZ, UK
Animal Health and Veterinary Laboratories Agency (AHVLA), AHVLA-Weybridge, Addlestone, Surrey KT15 3NB, UK

It is widely accepted that abnormal forms of the prion protein (PrP) are the best surrogate marker for the infectious agent of prion diseases and, in practice, the detection of such disease-associated (PrP\(\text{D}\)) and/or protease-resistant (PrP\(\text{R}\)) forms of PrP is the cornerstone of diagnosis and surveillance of the transmissible spongiform encephalopathies (TSEs). Nevertheless, some studies question the consistent association between infectivity and abnormal PrP detection. To address this discrepancy, 11 brain samples of sheep affected with natural scrapie or experimental bovine spongiform encephalopathy were selected on the basis of the magnitude and predominant types of PrP\(\text{D}\) accumulation, as shown by immunohistochemical (IHC) examination; contra-lateral hemi-brain samples were inoculated at three different dilutions into transgenic mice overexpressing ovine PrP and were also subjected to quantitative analysis by three biochemical tests (BCTs). Six samples gave ‘low’ infectious titres (10\(^{6.5}\) to 10\(^{6.7}\) LD\(_{50}\) g\(^{-1}\)) and five gave ‘high titres’ (10\(^{8.1}\) to \(>10^{8.7}\) LD\(_{50}\) g\(^{-1}\)) and, with the exception of the Western blot analysis, those two groups tended to correspond with samples with lower PrP\(\text{D}/\text{PrP}\text{R}\) results by IHC/BCTs. However, no statistical association could be confirmed due to high individual sample variability. It is concluded that although detection of abnormal forms of PrP by laboratory methods remains useful to confirm TSE infection, infectivity titres cannot be predicted from quantitative test results, at least for the TSE sources and host PRNP genotypes used in this study. Furthermore, the near inverse correlation between infectious titres and Western blot results (high protease pre-treatment) argues for a dissociation between infectivity and PrP\(\text{R}\).

INTRODUCTION

The detection of abnormal prion protein (PrP) accumulating in the brains of infected animals is the basis of the diagnostic tests for prion diseases or transmissible spongiform encephalopathies (TSEs). Whether by visualization of disease-associated PrP (PrP\(\text{D}\), also designated PrP\(\text{Sc}\)) aggregates by immunohistochemistry (IHC) or by detection of proteinase K (PK)-resistant PrP (PrP\(\text{R}\)) by biochemical tests (BCTs), demonstrating the presence of abnormal PrP remains the cornerstone of TSE diagnosis, and PrP\(\text{D}/\text{PrP}\text{R}\) is widely considered a surrogate marker of the infectious agent(s). However, early evidence from a variety of experiments (reviewed by Rohwer, 1991) suggests that relationships between infectivity and positive test results for PrP\(\text{D}/\text{PrP}\text{R}\) are not simple. More recently, in certain TSE models where infectivity has been demonstrated, little or no PrP\(\text{R}\) can be detected by conventional laboratory methods (Lasmezas et al., 1997; Barron & Manson, 2003; Barron et al., 2007; Balkema-Buschmann et al., 2011), while in other models the magnitude of PrP\(\text{D}\) revealed by IHC or histoblotting lacks correlation with infectivity (Jeffrey et al., 2001a). Such studies suggest the existence of infectious and pathological conformations of PrP that are not readily detectable by current diagnostic tests. Conversely, not all PrP\(\text{D}/\text{PrP}\text{R}\) forms that are
detected by laboratory tests methods may necessarily be infectious (Piccardo et al., 2007; Jeffrey et al., 2012), and dissociation between abundance of PrP<sup>res</sup> and infectivity has also been reported as one of the arguments against the prion hypothesis (Miyazawa et al., 2011).

Determining the association or dissociation between the TSE infectious agent(s) and its surrogate markers is of importance in terms of possible underdiagnosis, not only at the level of the animal but also in the identification of tissues that contain infectivity. Such determination is therefore of relevance for food safety, animal health and disease eradication policies.

In previous studies we have shown that, depending on the TSE strain and model, sheep diagnosed with clinical disease display different patterns of deposition of PrP<sup>d</sup> in the brain as demonstrated by IHC. For example, in the brains of CH1641 experimentally infected sheep, PrP<sup>d</sup> deposits are almost exclusively found within the cytoplasm of neurons and microglial cells (Jeffrey et al., 2006), while in a model of natural scrapie in Suffolk sheep, the majority of PrP<sup>d</sup> is extracellular and predominantly associated with astrocytes (González et al., 2002, 2003, 2010b; Sisó et al., 2010); by ultrastructural examination such extracellular PrP<sup>d</sup> is shown to aggregate at the cell membrane (Jeffrey et al., 2011). In further studies, sheep experimentally infected with the bovine spongiform encephalopathy (BSE) agent display conspicuous intra- and extracellular PrP<sup>d</sup> deposits (González et al., 2005a). These observations exemplify differences in morphology and cell-association of PrP<sup>d</sup> aggregates among different ovine TSEs. Differences in magnitude or levels of PrP<sup>d</sup> accumulation in the brain of clinically affected sheep are also demonstrable in different disease situations. Thus, sheep dosed orally with BSE have lower amounts of PrP<sup>d</sup> in the forebrain compared with sheep inoculated intracerebrally with the same inoculum and ARR/ARR sheep (A, alanine and R, arginine at codons 136, 154 and 171 of ovine PrP) challenged intracerebrally with BSE have little PrP<sup>d</sup> when compared with ARQ/ARQ sheep (Q, glutamine) challenged with the same inoculum and by the same route and at the same stage of clinical disease (González et al., 2005a). Similarly, VRQ/ARR sheep (V, valine) experimentally infected with the SSBP/1 scrapie source (González et al., 2002) and some naturally infected goats (González et al., 2010a; Konold et al., 2010), despite showing clinical signs of scrapie, display low magnitudes of brain PrP<sup>d</sup>. Indeed, when the IHC abundance of PrP<sup>d</sup> is determined for different models of TSE-infected sheep, there is no correlation between the amount of PrP<sup>d</sup> present, the incubation period and the presence of clinical disease. These observations raise questions about the nature of the infectious form of PrP and the efficiency of detection of different abnormal PrP forms and types by different test methods.

With these premises in mind, the present study questioned (i) whether a correlation exists between TSE infectivity levels in brain tissue of sheep, as determined by mouse bioassay, and levels of abnormal PrP, as detected by standard IHC and BCTs, and (ii) whether the levels of infectivity and test outputs from different PrP detection methods correlate with specific IHC types of PrP<sup>d</sup> accumulating in the brains of clinically affected, TSE-infected sheep.

**RESULTS**

**Correlation between BCTs**

For the 40 scrapie samples tested (see details in Methods), the three BCTs showed high correlation values, with Spearman <i>r</i> values of 0.70 for enzyme immunoassay (EIA) versus Western blot (WB), 0.72 for enzyme-linked immuno-sorbent assay (ELISA) versus WB and 0.72 for ELISA versus EIA (Fig. 1a). The correlations were also high for BSE samples, with <i>r</i> values between 0.69 (EIA vs WB) and 0.95 (ELISA vs EIA) for ARQ/ARQ sheep samples and between 0.74 (ELISA vs EIA) and 0.92 (ELISA vs WB) for ARR/ARR sheep samples (Fig. 1b). It is worth noting that ARR/ARR sheep BSE samples provided much lower values in all three BCTs than those from ARQ/ARQ sheep (Fig. 1b).

When analysing the association between BCT results independently for each brain area examined (Fig. 1 and details in Table S1, available in JGV Online), scrapie samples provided significant correlation values between all three BCTs in the cerebral cortex (0.77–0.95) and in the cerebellum (0.76–0.95), only between EIA and ELISA in the corpus striatum (0.65) and no significant correlations in the cerebellum (−0.05–0.48). For ARQ/ARQ BSE samples, correlations between the three BCTs were significant in the cerebellum (0.78–0.93), and only between ELISA and EIA in the cerebral cortex (0.89) and in the corpus striatum (0.41–0.86).

**Correlation between IHC and BCTs**

Significant correlations were obtained when comparing IHC scores for total PrP<sup>d</sup> and the results of the BCTs on the 40 scrapie brain samples examined (Table 1, Fig. 2a), with Spearman’s <i>r</i> coefficients of 0.41 for EIA, 0.52 for ELISA and 0.68 for WB. The magnitude of accumulation of all the different PrP<sup>d</sup> types recognized by IHC correlated well with the end-point dilution values obtained in the three BCTs with the exception of extracellular, astrocyte-associated PrP<sup>d</sup> (ASTR: subpial, peri-vascular, peri-vacuolar and subependymal types), which did not show correlation with any of the three BCTs. The stellate PrP<sup>d</sup> type (STEL; extracellular, possibly associated with resting microglia) gave the highest correlation coefficients with all three BCTs, followed by intracellular PrP<sup>d</sup> (ITCL: intra-neuronal, intra-microglial and intra-astrocytic types) and by extracellular PrP<sup>d</sup> in the grey matter neuropil (NRPL: particulate/coalescing, linear and peri-neuronal).

When the correlation between IHC and BCTs was analysed independently for the four brain areas (Table S1), similarly to what was observed when comparing the three BCTs, the
samples of obex did not provide significant correlation with either total PrP<sup>d</sup> or with the different types considered. Correlations were best in the cerebral cortex, where all four PrP<sup>d</sup> types considered correlated with all three BCT results, and where correlation with total PrP<sup>d</sup> was at its highest (0.84 for EIA to 0.94 for ELISA). Samples of cerebellum also provided high correlation coefficients, with the exception of ITCL PrP<sup>d</sup>, whereas in the corpus striatum, only WB provided significant correlation coefficients, again with the exception of ITCL PrP<sup>d</sup>. Notably, the highest correlation coefficients in all these brain areas were for the STEL PrP<sup>d</sup> type, in line with the values obtained in the overall analysis. In contrast, ASTR PrP<sup>d</sup>, which gave non-significant correlation in the global analysis, showed high correlation with the BCTs in the cerebral cortex, and less so in the cerebellum and corpus striatum.

Fig. 1. Correlation between BCTs. Results indicate end-point dilution values for each sample (scrapie, n=40; ARQ/ARQ BSE, n=21; ARR/ARR BSE, n=12) in the three different tests. Correlation coefficients [Spearman r (r<sub>s</sub>)] and their significance (**, P<0.01; *** P<0.001) are given for each comparison. Note that scales have been adjusted to actual values to provide a better representation of the correlations. For scrapie and ARQ/ARQ BSE samples open symbols indicate: circles, cerebral cortex; squares, corpus striatum; triangles, cerebellum; diamonds, obex (scrapie only). Black diamonds represent ARR/ARR BSE samples, regardless of brain area.
Table 1. Correlation analyses between IHC and BCTs in brain samples

P-values of correlation analyses are indicated as: NS, not significant; *, P<0.05; **, P<0.01; *** P<0.001. ITCL, Intracellular; STEL, stellate; ASTR, astrocyte-associated extracellular; NRPL, neuropil-associated extracellular (for extended description see text). Note that in the case of ARR/ARR BSE samples data are not shown for another type of PrPd, non-vascular plaques, which are nevertheless considered to provide the total PrPd values.

For the 33 BSE brain samples, the correlation obtained between IHC and BCTs was much lower than that observed for the scrapie samples (Table 1, Fig. 2b). The only correlations observed were between STEL PrPd and ELISA and EIA in ARQ/ARQ sheep, and between NRPL PrPd and all three BCTs and EIA and WB with ITCL PrPd in ARR/ARR sheep.

The analysis of ARQ/ARQ BSE samples by brain area confirmed the overall analysis insofar as the STEL type was the one showing the highest correlation coefficients in all three brain areas, although these were only significant for ELISA and EIA in the cerebral cortex and for EIA in the cerebellum (Supplementary data).

Correlation between laboratory test results and infectivity

Table 2 provides details of attack rates and incubation periods of the bioassay carried out in TgshpXI mice with the 11 brain samples selected on the basis of their PrPd profile and total magnitude of PrPd accumulation. Based on attack rates at the different dilutions the infectious titres were calculated, and it became clear that the sheep inocula could be divided in two groups: those that killed 90–100% of mice at the 10⁻³ and 0% at the 10⁻⁵ dilutions (n=6; titres 10⁻⁵–10⁻² LD₅₀ g⁻¹; 'low'), and those that killed 70–100% of mice at the 10⁻⁵ dilution (n=5; titres 10⁻⁴–10⁻¹ LD₅₀ g⁻¹; 'high').

Sheep samples of low infectious titres had an average lower magnitude of IHC-detectable PrPd (mean 5.4), and also gave lower ELISA (mean 466) and EIA (mean 1130) values than those with high infectious titres (mean values of 9.6, 779 and 2860 for IHC, ELISA and EIA, respectively); their WB results, however, were higher (mean 1821) than those of samples with high infectious titre (mean 781). None of those differences was significant in the Mann–Whitney, non-parametric test due to high individual variability of test results within each group (Figs 3 and 4). For IHC for example, while the three samples with the lowest magnitude of total PrPd [BSE1 Cc, Scra1 St (Fig. 4) and BSE2 Cc] were in the group with low infectious titre and the two with the highest PrPd scores [BSE6 Cc (Fig. 4) and BSE6 Cb] were in the group of high titre, samples with intermediate IHC scores could be in either group (Table 2 and Fig. 4).

A further analysis was performed to determine the relationship between infectivity and the scores of different PrPd types determined by IHC examination. Although the mean scores of all PrPd types considered were higher in the group of high than of low infectious titre, the only significant difference was with respect of the STEL PrPd type (Table 2 and Fig. 3).

DISCUSSION

A clear correlation between laboratory test results, that is levels of abnormal PrP detectable by different methods, and infectious titre, has not been demonstrated within this study. It is however true that, with the exception of WB, sheep brain samples containing higher levels of abnormal PrP tended to provide higher infectious titres, although the individual variability in laboratory test results made differences to be non-significant. In addition to the small sample size, a number of other considerations need to be made in order to discuss and interpret this somewhat poor correlation: the assessment of infectivity in TgshpXI mice was done at 100-fold dilutions, the highest being at 10⁻⁵; this created three problems that could have affected the discriminatory capability of the comparative analysis: (i) the bimodal rather than sequential distribution of infectious titles, which prevented the realization of correlation analyses, (ii) the close proximity (in some cases identity) of the titres of some samples, and (iii) the inability to calculate precise infectious titres in the two samples that resulted in 100% attack rate at the highest dilution. However, despite the fact that the infectious titres obtained partitioned into two distinct groups [considered to be of statistically significant different infectivity since titres differed by more than 0.8 log units (Mould et al., 1967)], no clear correlation was found with corresponding test results. Therefore, it is also likely that a more accurate calculation of titres would not have fundamentally changed their correlation with test results.

Because the selection of samples for the different analyses was done from archival material, the samples scored for PrPd by IHC were from one hemi-brain while those for biochemical analysis and bioassay were from the other hemi-brain.
Although it is widely assumed that both infectivity and normal PrP accumulation in the brain occur symmetrically, specific and detailed studies to verify these assumptions have not been carried out to the best of our knowledge. It is therefore possible that some asymmetry in the distribution of PrPd or infectivity might have contributed to a lack of clear correlation between IHC and infectious titres and between IHC and BCTs. This caveat, however, should not apply to the comparison between BCT results and infectivity, as all were carried out on the same ipsi-lateral brain samples. As the correlation between these tests and infectious scores was even lower than between infectivity and total amount of PrPd detected by IHC, it appears unlikely that poor correlations can be attributed to variations between tissue samples.

**Fig. 2.** Correlation between IHC and BCT results. Results indicate total PrPd scores (IHC) for each sample (scrapie, n=40; ARQ/ARQ BSE, n=21; ARR/ARR BSE, n=12) and their corresponding end-point dilution value in the three different BCTs. Correlation coefficients [Spearman r (r_s)] and their significance (no symbol, not significant; *, P<0.05; **, P<0.01; ***, P<0.001) are given for each comparison. Note that scales have been adjusted to actual values to provide a better representation of the correlations. For scrapie and ARQ/ARQ BSE samples open symbols indicate: circles, cerebral cortex; squares, corpus striatum; triangles, cerebellum; diamonds, obex (scrapie only). Black diamonds represent ARR/ARR BSE samples, regardless of brain area.
A possible explanation for the relatively discrepant results between infectivity and BCTs is that these tests, which detect either protease-resistant PrP fragments (ELISA and, particularly, WB) or abnormal PrP\textsubscript{d} in a particular state of aggregation (EIA), fail to detect, to a greater or lesser extent, infectious PrP conformers (if such are actually the infectious agent). The dissociation between infectivity and WB results argues for a separation between highly PK-resistant forms of PrP and infectivity. This is in contrast with reports from early studies (McKinley et al., 1983), but would be in agreement with others showing that partially disaggregated and PK-sensitive (Rohwer, 1991; Caughey et al., 1997), non-fibrillar PrP particles (Silveira et al., 2005) are associated with increased infectivity in hamsters. Along similar lines would be the reports of some cases of sporadic Creutzfeldt–Jakob disease, in which most or all the disease-associated PrP\textsubscript{d} is PK-sensitive (Safar et al., 2005; Gambetti et al., 2008; Head et al., 2009). The better association between the ELISA (low PK concentration) or the EIA (no PK treatment) results and infectious titres would support the notion of infectivity being related with partially PK-sensitive, not highly aggregated forms of abnormal PrP. Furthermore, the fact that our IHC protocol did not use enzyme digestion and is able to detect a wider range of PrP\textsubscript{d} forms in different aggregation states and of different PK resistance (González et al., 2005b), could have contributed to the better association found between IHC and infectivity.

With regard to the relationship between different morphological PrP\textsubscript{d} types recognized by IHC and infectivity, the only type that showed significant correlation was the stellate type and, to a lesser, non-statistically significant extent, the intracellular PrP\textsubscript{d}. When these two types are combined, the difference between the low infectious score group (\(n=6\), mean \(\pm\) SEM of combined ITCL + STEL PrP\textsubscript{d}=2.4 \(\pm\) 0.81) and the high infectious score group (\(n=5\), 5.1 \(\pm\) 0.97) is not statistically significant (\(P=0.063\) in the \(t\)-test). The apparent correlation between infectivity and stellate PrP\textsubscript{d} may not be biologically relevant; while

### Table 2. Individual results of infectious titre and laboratory test results of brain samples taken for bioassay

<table>
<thead>
<tr>
<th>Sheep</th>
<th>171</th>
<th>Area</th>
<th>Titre</th>
<th>ITCL</th>
<th>STEL</th>
<th>ASTR</th>
<th>NRPL</th>
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<tbody>
<tr>
<td>BSE1</td>
<td>RR</td>
<td>Cc</td>
<td>6.5</td>
<td>0.7 (0)</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Scra1</td>
<td>QQ</td>
<td>St</td>
<td>6.7</td>
<td>0.1 (0.1)</td>
<td>0.2</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>BSE2</td>
<td>QQ</td>
<td>Cc</td>
<td>6.7</td>
<td>0.6 (0.3)</td>
<td>0.7</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Scra2</td>
<td>QQ</td>
<td>Ob</td>
<td>6.7</td>
<td>3.2 (2)</td>
<td>2</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>BSE3</td>
<td>RR</td>
<td>Ob</td>
<td>6.7</td>
<td>6 (1.5)</td>
<td>0.2</td>
<td>0.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Scra3</td>
<td>QQ</td>
<td>Cc</td>
<td>6.7</td>
<td>0.5 (0)</td>
<td>1.2</td>
<td>6.9</td>
<td>1.1</td>
</tr>
<tr>
<td>BSE4</td>
<td>QQ</td>
<td>Cc</td>
<td>8.1</td>
<td>1.3 (0)</td>
<td>1.2</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>BSE5</td>
<td>QQ</td>
<td>Cb</td>
<td>8.4</td>
<td>2.8 (1)</td>
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<td>1</td>
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<tr>
<td>BSE6</td>
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<td>Cb</td>
<td>8.4</td>
<td>5.5 (1)</td>
<td>3</td>
<td>3.2</td>
<td>5</td>
</tr>
<tr>
<td>BSE1</td>
<td>RR</td>
<td>St</td>
<td>(\geq 8.7)</td>
<td>3.6 (1)</td>
<td>1.1</td>
<td>0.6</td>
<td>3.2</td>
</tr>
<tr>
<td>BSE6</td>
<td>QQ</td>
<td>Cc</td>
<td>(\geq 8.7)</td>
<td>3 (0.2)</td>
<td>2.2</td>
<td>5.1</td>
<td>0.1</td>
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</table>

CC, Cerebral cortex; St, corpus striatum; Ob, obex; Cb, cerebellum. AR, attack rate (%); IP, incubation period in mean days \(\pm\) SEM (superscripts indicate dilution). Titres expressed as log\textsubscript{10} of mouse (intracerebral) LD\textsubscript{50} g\textsuperscript{-1}. ARs and IPs are not given for the 10\textsuperscript{-1} dilution of the different samples (ARs of 100% in all cases and mean IPs between minimum 248 days for BSE5 and maximum 474 days for Scra2). ITCL, Intracellular (in parentheses intraneuronal PrP\textsubscript{d} scores); STEL, stellate; ASTR, astrocyte-associated extracellular; NRPL, neuropil-associated extracellular (for extended description see text).
intracellular, or at least intraneuronal, PrPd is commonly found in sheep BSE and classical scrapie cases, the stellate pattern is not a universal feature of sheep TSEs. For instance, experimental CH1641 scrapie infection lacks stellate PrPd (Jeffrey et al., 2006), indicating that this PrPd type can at best provide only partial correlation with infectivity.

It has been recently reported that hamsters injected with the ‘hyper’ strain of transmissible mink encephalopathy show short survival times and presence of intraneuronal PrPSc, while those challenged with the ‘drowsy’ strain of the same agent have considerably longer incubation periods and absence of intraneuronal PrPSc deposits (Ayers et al., 2011). The authors suggested that such differences in incubation period are due to differences in PrPd processing within and subsequent damage to neurons. Another explanation could be that brains with high levels of intraneuronal PrPd are more infectious than those without such PrPd type. The results of our study, however, are inconsistent with the second possibility since sheep brain samples with low infectious titres could either show conspicuous intraneuronal PrPd deposits [e.g. BSE3 Ob (Fig. 4e) and Scra2 Ob] or none at all [e.g. Scra3 Cc (Fig. 4f) and BSE1 Cc (Fig. 4a)], and vice-versa (Table 2).

Some unanticipated observations have arisen from the analyses of the laboratory test results. Firstly, correlations between BCT results appeared to be brain area-dependent, at least for scrapie, in which such correlations did not exist in samples of obex but were consistently significant in the cerebral cortex and cerebellum. Also, both for scrapie and BSE in ARQ/ARQ sheep, ELISA and EIA provided better correlation values between them than when compared to WB. This was not the case, however, for ARR/ARR BSE samples for which WB showed the highest correlations. Secondly, from the comparative analysis of IHC profiles and BCTs, it would appear that the different morphological types of PrPd that can be identified in the brains of sheep may have different biochemical properties, but that these are also brain area- and agent-dependent. Thus, in ARQ/ARQ BSE sheep, ASTR extracellular PrPd did not correlate with BCT results in any of the brain areas examined, while it did so in the cerebral cortex, and to a lesser extent in the cerebellum and corpus striatum, of scrapie-infected sheep. In contrast, in sheep of the same ARQ/ARQ genotype, STEL PrPd was the type that provided the highest and most consistent correlations across different brain areas regardless of the infectious agent. Taken together, these observations suggest different morphological and cell-associated PrPd types may have different molecular properties in terms of aggregation states or resistance to protease treatment and denaturation, but that these are also influenced by a complex combination of infectious agent, host genetics and brain topography. Since all brains examined in this study were from animals at clinical end-point and since the different brain areas start accumulating PrPd/PrPSc at different times after infection [obex, cerebel- lum, corpus striatum and cerebral cortex from earliest to latest (Sisó et al., 2009)], it is also possible that alterations in molecular properties of PrPd/PrPSc are time-dependent.

The high correlation found between the three BCTs employed is not surprising; these tests had been previously evaluated in a European Food Safety Authority trial and two of them, the ELISA and the EIA, gave similar diagnostic and analytical sensitivity on small ruminant TSE samples (EFSA Panel on Biological Hazards, 2009). Moreover, the three BCTs were performed on identical tissue homogenates, which undoubtedly contributed to the high correlation of their results. Considering that two of them, ELISA and WB, use PK digestion, while EIA relies on a polyanionic ligand-affinity and detects aggregated PrPd, the high correlation between the three BCTs argues for an association between protease resistance and aggregation state.

In conclusion, PrPd/PrPSc accumulating in the brains of sheep infected with scrapie or BSE, as detected by IHC and BCTs, is still a useful surrogate marker for infection or diagnosis of clinical disease. However, in quantitative terms – and at least
within the context of the TSE sources and host PRNP genotypes used in this study – it does not necessarily reflect the extent of replication of the infectious agent, so that infectious titres cannot be predicted from laboratory test results.

**METHODS**

**Animals and samples.** Tissue samples from 20 sheep from two different, unrelated studies were used in the present study. These were: (i) 10 ARQ/ARQ Suffolk sheep clinically affected with natural scrapie, originating from a closed flock [Moredun Research Institute (MRI)]. Details of the clinico-epidemiological aspects of the infection and of the pathological and 
PrP\(_d\) IHC characteristics of the disease in this flock have been extensively documented elsewhere (Jeffrey et al., 2001b; González et al., 2002, 2010b; Siso et al., 2010). Nine of the 10 sheep selected for this study were born in 2002 and one in 2000; all of them (seven females and three males) had reached clinical end-point of scrapie at 702–860 days of age and all showed indistinguishable PrP\(_d\) profiles by brain IHC. (ii) Seven ARQ/ARQ and three ARR/ARR Romney sheep intracerebrally inoculated with a sheep-derived BSE inoculum (González et al., 2007). Three, three and one of the ARQ/ARR sheep (five males and two females) had been dosed with \(10^{-3}\), \(10^{-4}\) and \(10^{-5}\) dilutions of the original brain homogenate, respectively, and had reached clinical end-point of laboratory-confirmed BSE at 476–562, 499–604 and 828 days post-infection (p.i.), respectively. The three ARR/ARR sheep (two females and one male) had been inoculated with a \(10^{-3}\) dilution of the same brain homogenate and reached BSE clinical end-point at 1414–1710 days p.i.

None of the 17 ARQ/ARQ sheep showed polymorphisms at any codon of the PRNP gene.

From each of the 20 sheep, samples of frontal cerebral cortex, corpus striatum, cerebellum and medulla oblongata at the obex from the left hemi-brain were fixed in formaldehyde and processed for IHC examinations. Tissue samples from the same areas of the right hemi-brain

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**Fig. 4.** IHC appearance of PrP\(_d\) in samples with different infectious titres. Some samples show correlation between magnitude of PrP\(_d\) and titre: (a) (BSE1 Cc) and (c) (Scra1 St) show little PrP\(_d\) (1.2 and 1.0, respectively) and low infectivity (log titres of 6.5 and 6.7, respectively); (b) (BSE1 St) and (d) (BSE6 Cc) show moderate/high PrP\(_d\) levels (9.5 and 10.4, respectively) and high infectious titres (log titre \(\geq 8.7\) in both cases). Other samples, however, do not show such correlation: (e) (BSE3 Ob) and (f) (Scra3 Cc) show similar PrP\(_d\) magnitudes (9.1 and 9.7, respectively) to samples illustrated in (b) and (d) but their infectivity is low (log titre \(= 6.7\) in both cases); despite identical infectious titre, note also the difference in intraneuronal PrP\(_d\), which is notably more prominent in BSE3 Ob (e) than in Scra3 Cc (f; for values refer to Table 2). IHC with R145 PrP antibody; magnifications: (a), (d), (f) \(\times 10\); (b), (c) \(\times 20\), (e) \(\times 4\).
(except for obex, which was not available for the seven BSE-affected ARQ/ ARQ sheep) were kept frozen at −80 °C for BCT analyses. Therefore, a total of 73 brain samples, 40 from scrapie- and 33 from BSE-affected sheep, were available for comparison between IHC features and BCT results.

Eleven of the 73 brain samples, three from scrapie and eight from BSE-affected sheep, were selected for bioassay in TgshpXl transgenic mice, which overexpress ovine ARQ/ARQ PrP (Kupfer et al., 2007). The selection of the samples was made on the basis of differences in magnitude of PrP\textsuperscript{d} accumulation observed by IHC (scores from 1.0 to 16.7) and/or in the proportion of different morphological and cell-associated PrP\textsuperscript{d} types.

**IHC and BCT in sheep brain samples.** The processing of brain samples for IHC, which was performed with PrP antibody R145 (AHVLA), and the scoring system for quantification of PrP\textsuperscript{d} accumulation have been described in detail previously (González et al., 2002, 2005a). Briefly, the magnitude of different types of disease-associated (PrP\textsuperscript{d}) aggregates was scored from 0 to 3 in the 73 samples included in this study. To facilitate comparisons with BCT and infectivity titration results, the PrP\textsuperscript{d} types were grouped as follows: (i) intracellular (ITCL): intra-neuronal, intra-microglial and intra-astrocytic, for a maximum score of 9; (ii) stellate (STEL) for a maximum score of 12; (iv) grey matter neuropil-associated (NRPL): particulate, perivascular, subependymal and peri-vacuolar, for a maximum score of 3; (iii) astrocyte-associated (ASTR): subpial, peri-astrocytic, for a maximum score of 9.

The only other type of PrP\textsuperscript{d} observed in the samples examined were non-vascular plaques, exclusively found in the three BSE-infected ARR/ARR sheep; this type is not considered in the analyses of individual PrP\textsuperscript{d} types but was included in the score for total PrP\textsuperscript{d} in each sample, which was worked out as the sum of scores of the different types considered (maximum theoretical score of 36). Since the IHC protocol did not include a protease-treatment step, PrP\textsuperscript{d} is putatively composed of both protease-resistant and protease-sensitive forms of abnormal, disease-associated PrP (González et al., 2005b).

Three BCTs were applied to the 73 selected brain samples. Two of the tests, the TeSeE ‘sheep and goat’ (Bio-Rad) and the Bio-Rad TeSeE ‘sheep and goat’ Western blot analysis (WB), are based on immunoaffinity and relative PK resistance at two different PK concentrations (fivefold lower in the ELISA than in the WB), thus detecting PrP\textsuperscript{d}. The other, IDEXX HerdChek BSE/scrapie enzyme immunoassay (EIA; Idexx Laboratories), is a polyanionic ligand-affinity based method and does not require PK treatment, thus detecting PrP\textsuperscript{d}. All three BCTs enable quantification of the abnormal PrP in the samples by means of end-point dilution assays, the results of which were used for comparison with the IHC scores and the titration results in the bioassay. Dilution curves for each sample were achieved by serially diluting samples 1:2 to give a dilution range from neat to 1:32 768. ELISA and WB samples were diluted post-extraction using sample diluent (R6) and Laemmli solution, respectively and then analysed according to manufacturer’s instructions. EIA samples were diluted using de-ionized water prior to the addition of working plate diluent; once diluted all were analysed following manufacturer’s instructions with diluted samples treated exactly the same as neat samples. We had previously shown that there were no noticeable differences between EIA dilution curves prepared using negative control brain homogenate [25% (w/v) in kit homogenization buffer] and water (data not shown). End-point dilutions were given as the dilution at which the absorbance reading for the test sample was the same as that for the test kit negative control (ELISA and EIA) or the last dilution at which PrP\textsuperscript{d} bands were observed (WB).

**Bioassay in TgshpXl mice.** Groups of 10 TgshpXl mice were each inoculated intracerebrally with 10^{-1}, 10^{-3} and 10^{-5} dilutions of each of the selected 11 sheep brain samples. This transgenic mouse line was selected because of Prnp genotypic homology with most of the samples inoculated; conventional wild-type mice were disregarded in view of previous evidence of inefficient transmissibility of scrapie isolates from the MRI Suffolk flock to such mice (Bruce et al., 2002). Mice reaching clinical end-point of neurological disease compatible with scrapie/BSE, and those showing significant mobility deterioration or inability to eat or drink, were euthanized with carbon dioxide. Brains were removed and placed in buffered formalin and confirmation of scrapie/BSE was carried out by IHC for PrP\textsuperscript{d} with rabbit polyclonal R486, which recognizes amino acid sequence 221–233 of bovine PrP, by procedures described elsewhere (Beck et al., 2010). All mouse procedures were performed in compliance with the Animal (Experimental Procedures) Act 1986 under licence no. 70/6310 issued by the UK Home Office and were approved by the local ethics committee.

Infectious titres (LD\textsubscript{50}) were determined by the Spearman–Karber formula (Hamilton et al., 1977): \[ \log_{10}\text{LD}_{50} = (X_0 - [d/2]) + d \times \log_{10}(n_i/n_i) \]

where for this case:

\[ X_0 = \log_{10} \text{of the lowest dilution at which all mice died of scrapie or BSE} \]

\[ d = \text{dilution factor} \]

\[ n_i = \text{number of positive mice in the X}_0 \text{ and subsequent dilutions} \]

\[ n_i = \text{number of mice inoculated in the X}_0 \text{ and subsequent dilutions} \]

(discourting those dying from intercurrent deaths)

Since 20 µl of inocula were used for the intracerebral inoculations and the LD\textsubscript{50} is expressed by gram of tissue, a conversion factor of \times 500 was applied to the titres obtained.

**Statistical analyses.** The relationship between the results of the three BCTs on individual samples (end-point dilution values) was assessed by means of Spearman’s non-parametric correlation analyses (Instat GraphPad Software). The same approach was used to compare between individual sample results in IHC and in each of the three BCTs. These analyses were done for the total number of samples (n=40 in scrapie, n=21 in ARQ/ARQ BSE and n=12 in ARR/ARR BSE samples) regardless of brain area of provenance, and also for each individual area separately. The later were not performed on ARR/ARR BSE samples due to the low number of sheep involved (n=3).

The relationship between infectivity and laboratory test results was assessed by Mann–Whitney non-parametric analysis applied to samples grouped according to their titre. This strategy was necessary in view of the bi-modal distribution of the infectious titres obtained in the 11 samples selected for bioassay.

**ACKNOWLEDGEMENTS**

This study was funded by Defra project SE2007, with sheep samples used for bioassay and laboratory tests coming from projects SE1842 (BSE) and SE1949 (scrapie), also funded by Defra. The authors are indebted to L. Fairlie, A. Dunachie and M. Oliva (AHVLA-Lasswade) for technical support on sheep and mouse immunohistochemistry and to the staff in the Animal Service Unit (ASU-Weybridge) and in the Moredun Research Institute for mouse and sheep up-keeping and post-mortems, respectively. We also thank Martin Groschup (Friedrich-Loeffler Institute) for provision of TgshpXl mice and to Jim Hope (AHVLA) for critical and constructive reading of the manuscript.

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