Partial dissociation of PrPSc deposition and vacuolation in the brains of scrapie and BSE experimentally affected goats

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The diagnosis of transmissible spongiform encephalopathies (TSEs), including natural scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jacob disease of man, can be characterized by the formation of the disease-associated isoform of the prion protein (PrPSc). During the course of an investigation, goats clinically affected following experimental infection with three different sources of TSE (SSBP/1, CH1641 and BSE) developed widespread vacuolar degeneration in the brain. With BSE, PrPSc was clearly recognized in affected goat brain by immunocytochemistry (icc) and Western blotting, but in contrast the experimental scrapie sources SSBP/1 and CH1641 showed almost no or very little PrPSc by icc. Western blot analysis of PrPSc from BSE-affected and SSBP/1-affected goat brain showed that the protein was present in brain affected by both TSE sources, but could not be used to determine how much protein was present. It became clear that PrPSc and vacuolation could be partially dissociated following challenge with two of the three TSE sources. Subtle differences in glycosylation patterns between BSE- and SSBP/1-associated PrP protein isoforms could also be recognized, although these experimentally generated results should not be regarded as a BSE/scrapie differential test. However, our study warns that the reliance on PrPSc determination by icc alone as a means by which to diagnose TSE infection may generate false negative results.

Introduction

The transmissible spongiform encephalopathies (TSEs), including natural scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jacob disease of man, can be characterized by the formation of the disease-associated isoform of the prion protein (PrP), usually referred to as PrPSc (Bolton et al., 1982; McKinley et al., 1983). PrPSc is partially resistant to protease treatment and has been linked with scrapie infectivity, in contrast to the normal form of PrP (PrPC), which is completely digested by proteases and is found in both TSE-affected and normal healthy individuals (Prusiner et al., 1983). It is often stated that accumulation of PrPSc in brain and other tissues can be used instead of time-consuming and expensive bioassays for establishing the presence of TSE infection (Collinge et al., 1996). In addition, since PrP protein fragments have been shown to be neurotoxic in vitro (Brown et al., 1996), it is tempting to suggest that it is the accumulation of PrPSc which is directly related to the vacuolation and death of neurons seen in clinically affected animals.
CH1641 and SSBP/1) and scrapie is a natural disease of goats, which makes them a most suitable model to examine TSE biological characteristics. It is also theoretically possible that, like cattle, UK goats could have become infected by BSE through contaminated feed and, therefore, the characteristics of BSE in goats are of interest.

In the present study, brain sections from goats which had been challenged intracerebrally with the SSBP/1 and CH1641 scrapie sources and with BSE, were examined for colocalization of vacuolation and the presence of disease-associated PrP using ICC. Western blotting analysis of PrP<sup>Bsc</sup> (treated with proteinase K) from the brains of SSBP/1- and BSE-infected goats was also carried out to compare the PrP<sup>Bsc</sup> protein banding patterns resulting from different ratios of the mono-, di- and un-glycosylated forms of the protein (glycoforms) (Hope et al., 1999).

### Methods

#### Goats and sheep.

Goats were of mixed ancestry of the breeds Anglo–Nubian, Toggenburg and Saanan. They were between 370 and 2560 days old (Table 1) when challenged under anaesthesia by intracerebral injection into the right parietal cortex with a 0.5 ml volume of a 10% brain homogenate (equivalent to 0.05 g brain) of one of three TSE isolates. Challenged goats were kept at pasture year-round and only fed supplementary hay and/or vegetable-based protein supplement through the winter. The animals were challenged as part of various experiments and publications are referenced where appropriate.

Sheep used for bioassay were South Country Cheviots from the NPU flock and carried the PrP genotype VRQ/ARR (see later under genotypes), which does not contract natural scrapie in this flock (Hunter et al., 1996). They were challenged by subcutaneous inoculation into the hind legs with a 10% brain suspension.

#### Sources of inoculum.

The following TSE sources were used in this study. SSBP/1 originated as an homogenate of three natural scrapie brains that were subsequently passaged mostly through NPU Cheviot sheep (Dickinson et al., 1989) with incubation times controlled by PrP genotype at codon 171 (Goldmann et al., 1994). SSBP/1 also produces disease in goats, although its linkage to PrP genotype has not been established (Goldmann et al., 1996). For this study, eight goats challenged with SSBP/1 were examined.

The CH1641 isolate was recovered from a natural scrapie case in the NPU Cheviot sheep flock and generates distinctive incubation periods (Foster & Dickinson, 1988) related to PrP genotype at codon 171 (Goldmann et al., 1994). CH1641 produces disease in goats related to genotype of the PrP gene at codon 142 (Goldmann et al., 1996). Included in this study were three goats challenged with CH1641.

BSE infectivity was in the form of an homogenate of four terminally BSE-affected cattle brains. Diagnosis of all four cases was confirmed by histopathology and the transmission to mice from each BSE brain produced a pattern of disease which was characteristic of BSE (Fraser et al., 1992; Bruce et al., 1994). BSE has been successfully transmitted to goats

### Table 1. Incubation periods and age at challenge of goats inoculated by intracerebral injection with three TSE sources

<table>
<thead>
<tr>
<th>TSE source (no. of animals)</th>
<th>Animal no./age at challenge (days)</th>
<th>Incubation periods (days)</th>
<th>Mean incubation periods ± SE (days)</th>
<th>Western blot lane (see Fig. 2)</th>
<th>Vacuolation score*</th>
<th>Icc score*</th>
<th>Fig. 1 showing levels of vacuolation/icc</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSBP/1 (8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>37 × 33 (1395)</td>
<td>565</td>
<td>649 ± 47</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>48 × 83 (700)</td>
<td>567</td>
<td></td>
<td>1</td>
<td>0</td>
<td>3</td>
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<td>37 × 39 (1394)</td>
<td>574</td>
<td></td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<tr>
<td>48 × 82 (700)</td>
<td>596</td>
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<td>1</td>
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<tr>
<td>47 × 00 (711)</td>
<td>659</td>
<td>457 ± 119</td>
<td>6</td>
<td>1</td>
<td>5</td>
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<td>47 × 02 (711)</td>
<td>659</td>
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<td>CH1641 (3)†</td>
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<td>J0954 (370)</td>
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<td>1</td>
<td>4</td>
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<tr>
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<td>2</td>
<td>0</td>
<td>4</td>
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<td>3</td>
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<td>BSE (8)‡</td>
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<td></td>
<td></td>
<td></td>
<td>11</td>
<td>0</td>
<td>4</td>
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<tr>
<td>45 × 45 (2560)</td>
<td>547</td>
<td>665 ± 70</td>
<td>9</td>
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<td>4</td>
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<tr>
<td>45 × 49 (2560)</td>
<td>547</td>
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<td>0</td>
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<td>45 × 50 (1200)</td>
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<td>7</td>
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<td>4</td>
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<td>2</td>
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<tr>
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<td></td>
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* Scoring areas for vacuolation and immunocytochemistry: 1, dorsal vagus nucleus; 2, thalamic nuclei; 3, parietal cortex.
† Incubation period data from Foster & Dickinson (1988).
‡ Incubation period and PrP genotype data from Goldmann et al. (1996) and Foster et al. (1999). 506 and 570 incubation periods from Foster et al. (1993).
by experimental challenge (Foster et al., 1993) with incubation periods related to PrP codon 142 genotype (Goldmann et al., 1996). This study included six goats which had been challenged with BSE as part of an embryo transfer study (Foster et al., 1999) and two which had been used for strain-typing BSE (Foster et al., 1993).

**Histology and immunocytochemistry.** Brain recovered at post-mortem was fixed by immersion in formal saline and processed according to a standard protocol (Stembelger et al., 1970). Paraffin sections (6 µm) were cut coronally to include the cuneate and dorsal vagus nuclei, pontine nuclei, mesencephalon, diencephalon, basal ganglia, parietal and frontal cortex. Sections for histology were stained with haematoxylin and eosin (H&E).

Sections for immunohistochemistry were pre-treated with 98% formic acid for 5 min, and for 20 min with 0-1% trypsin at 37 °C in Tris–HCl buffer pH 7-6, to enhance PrP immunolabelling. They were immunostained using the indirect two-step method with the primary antibody as a mouse monoclonal raised against either ovine recombinant PrP protein N-terminal sequence (FH11 at 1:3; Foster et al., 1996) or bovine recombinant PrP protein (BG4 at 1:3; Foster et al., 1999) and the secondary antibody conjugated to peroxidase. The polyclonal antibodies 1A8 and 1B3 were raised in rabbits to mouse-passaged ME7 PrP, and used for comparative immunostaining (1:200; Jeffrey et al., 1994). The antigenic amino acid sequences of the PrP protein with which both FH11 and BG4 react are numbered 47–57 and 89–99 (C. Birkett, personal communication), while the sequences of the PrP protein with which both FH11 and BG4 react are numbered 14–36, 83–102, 119–139 and 188–212 (Langeveld et al., 1994).

Diaminobenzidine (brown) or aminoethylcarbazole (red) were used as substrates and all washes were performed with PBS buffer containing 0.2% BSA. Because no proteinase K is used in this method, positive staining is referred to as ‘disease-associated PrP’c. In effect virtually all PrPc is removed during tissue processing and following the pretreatments of formic acid and trypsin, which means that all immunostaining is really PrPsc.

The scoring system used to evaluate the intensity of both vacuolation and PrP immunostaining with icc is a previously reported subjective assessment by one of the authors (Foster et al., 1996) and is based on the range 0 to 5, where 5 is heavily vacuolated or stained (see Fig. 1).

**PrP protein extraction and Western blotting.** At post-mortem, tissues for PrP protein extraction were flash frozen in liquid nitrogen and stored at −70 °C until required. No frozen tissue was collected from CH1641 cases because these challenge experiments culminated in the early 1980s, prior to the realization that tissue samples would be needed for PrP detection. PrPc and PrPsc protein was analysed by Western blotting, performed following the methods of Hope et al. (1986), and the rabbit polyclonal antibody 1B3 was used for immunolabelling PrPsc. Each track of the blotting gel was loaded with between 0·1 and 0·15 g of tissue from either SSBP/1 or BSE brain material.

**Genotypes.** PrP genotypes generated as reported previously (Goldmann et al., 1994, 1996, 1998) use the one-letter amino acid code: V, valine; A, alanine; Q, glutamine; R, arginine; L, isoleucine; M, methionine; G, glycine; W, tryptophan, with subscripts giving the codon numbers. The octapeptide repeat polymorphism is indicated by repeat numbers, 5 or 3. All goats were of repeat genotype 5/5 and were WW112 unless stated otherwise.

**Results**

Brain pathology and infectivity

Eight goats injected with SSBP/1 developed clinical signs, which consisted predominantly of a rapidly progressive incoordination over 2–3 days. Different ages of the animals at challenge did not have any effect on incubation period in these studies (Table 1). Seven goats (PrP genotype 5/5 WW112 II112) had to be culled at between 500 and 600 days after challenge (Table 1; Goldmann et al., 1996) and the eighth goat (PrP genotype 3/5 WG102 II142) had an incubation period of 968 days (Goldmann et al., 1998). Histological assessment of brain from all eight SSBP/1 cases, regardless of PrP genotype or incubation period, showed intensive vacuolation particularly in the lateral thalamic nuclei (Fig. 1a). Medial and ventral nuclei were relatively less severely affected, as was the hypothalamus. Vacuolation was also present in the tectum, septum, basal ganglia, parietal cortex and some nuclei of the medulla oblongata, e.g. raphe, cuneate, dorsal vagus and olivary, although with slight variation between cases.

Six goats injected with BSE had incubation periods of 506 to 608 days with the seventh and eighth having incubation times of 982 and 985 days, which was linked to PrP genotype (IM114). Eight goats injected with CH1641 showed a distribution of vacuolation broadly similar to that observed with SSBP/1 (Fig. 1c, e, g) with the most severe vacuolation to be found in the tectal and thalamic areas (Foster et al., 1999).

Infectivity was confirmed in the brain (thalamus) of one of these SSBP/1-infected goats (incubation of 596 days) when transmission was achieved by bioassay via subcutaneous inoculation into two NPU Cheviot sheep of PrP genotype VA136 QR171 with incubation periods of 370 and 473 days. These incubations are well within expected limits for this sheep PrP genotype challenged with SSBP/1 (Goldmann et al., 1994) and indicate, therefore, that infectivity levels in the brain of this donor goat are equivalent to those in terminally affected SSBP/1-challenged sheep. VA136 QR171 genotype sheep are also known not to develop natural scrapie in the NPU flock (Hunter et al., 1996) and so the transmissions are genuine. The vacuolation profiles for both bioassay sheep in the medulla oblongata showed almost no signs of degeneration and were typical of SSBP/1 in sheep (Foster et al., 1996). The 473 day bioassay case, however, exhibited slightly elevated levels of vacuolation in thalamic and cortical regions.

**Detection of PrP by immunocytochemistry**

Immunocytochemistry using the monoclonal antibodies BG4 and FH11 or the polyclonal antibodies 1A8 and 1B3 produced no or very little PrP immunostaining throughout the brains of goats inoculated with SSBP/1 or CH1641, even in areas where vacuolation was most extensive (Fig. 1b; Table 1). Four from eight goats challenged with SSBP/1 did exhibit a very mild, extracellular punctate staining in the thalamus; however, this was not related to PrP genotype (Fig. 1b). In one of the latter cases immunostaining was quite widespread,
Fig. 1. (a) H&E section showing extensive vacuolation (score 5) in thalamic nuclei from goat 47 × 00 with terminal scrapie 659 days following intracerebral inoculation with SSBP/1 scrapie (× 200). (b) Immunohistochemical staining for PrP with BG4 antibody in thalamic nuclei of goat 47 × 00 showing marginal PrP staining (score ≤ 1) (× 200). (c) H&E section showing
but light, throughout the thalamic nuclei, while the other three cases showed only localized focal staining. In the remaining four goats, disease-associated PrP either could not be recognized or was present as a few grains in each of the brain areas scrutinized. Only one of the three goats inoculated with CH1641 demonstrated any PrP immunostaining. This was restricted to a few grains of peri-neuronal decoration in the thalamus and was not related to PrP genotype or incubation period (Fig. 1f). In contrast, goats challenged with BSE exhibited (with antibody BG4) much stronger disease-associated PrP immunostaining, which was prominent in the thalamic (Fig. 1d; Table 1) and hypothalamic nuclei (Fig. 1h; Table 1), and in the basal ganglia.

Disease-associated PrP was also detected by icc in some thalamic and cortical areas of the brains of the two sheep used as bioassays for SSBP/1-challenged goat brain, but was absent or scarce in the medulla.

**Detection of PrP by Western blotting**

Despite the apparent absence of PrPSc in the SSBP/1 icc, PrPSc was detectable by Western blotting, using the 1B3 antibody, of brain from seven of the eight SSBP/1-infected, and all eight of the BSE-infected goats (Fig. 2). The eighth SSBP/1-infected goat, which was negative for PrPSc by Western blotting, was also negative by icc. Proteinase K-treated SSBP/1 samples had less intense signals than equivalently treated BSE samples; however, this method is not designed to provide a direct quantification of the amount of PrP in each sample, it simply shows presence or absence.

Sample sizes of brain for both TSE sources were between 0.1 and 0.15 g of tissue per animal recovered from as close to the area of the thalamus as possible. There were slight differences in the PrPsc banding pattern between the sources. BSE samples showed predominantly the di- and monoglycosylated isoforms of PrPSc, whereas the SSBP/1 samples, although there was some individual variation, also showed the presence of the nonglycosylated, lower molecular mass protein band. This feature of PrPSc (or PrPSc) has been noted previously in samples from BSE-affected individuals (Collinge et al., 1996; Hope et al., 1999).

Western blotting has also been carried out on goat and sheep experimental BSE and scrapie brain samples using the three other antibodies described for icc (BG4, FH11, IA8). With IA8, PrPSc detection levels were similar to those revealed by 1B3; however, non-specific background staining levels were much higher with IA8. BG4 was slightly less sensitive, especially on scrapie PrP, whilst FH11 detection of proteinase K-treated PrP was very poor.

**Discussion**

This study demonstrates a partial dissociation between vacuolation and the detection by icc of the disease-associated PrPSc protein in goats inoculated with two distinct isolates of scrapie, namely SSBP/1 and CH1641. Intensive vacuolation occurred in the thalamic nuclei of goats affected by both scrapie sources, while immunostaining of PrPSc was only barely detectable. In direct contrast with both scrapie isolates, immunostaining of PrPSc was easily achieved in goats inoculated with BSE showing the intensity and distribution of vacuolation similar to that produced by SSBP/1 and CH1641. This ability to identify PrPSc by icc in the goat BSE experimental model (Foster et al., 1999) indicates that low

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**Fig. 2.** Western blots of proteinase K-treated (+) or untreated (−) brain samples from clinically affected goats which had been challenged intracerebrally with either SSBP/1 scrapie or BSE. The 1B3 polyclonal antibody was used for immunodecoration and each lane was primed with 0.1 g of brain equivalent. Animals numbered 1 (37 × 39), 2 (37 × 34), 3 (47 × 08), 4 (37 × 33), 5 (47 × 02) and 6 (47 × 00) were all inoculated with SSBP/1, while 7 (45 × 47), 8 (45 × 48), 9 (45 × 49), 10 (45 × 50), 11 (45 × 45) and 12 (45 × 46) were injected with BSE.

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levels of detection of PrPSc are not simply a feature of goats, regardless of the infecting source of TSE. In a similar study of PrP immunostaining with either natural or experimental scrapie in sheep, PrPSc could be observed without evidence of vacuolation in the vicinity, but rarely was the reverse found (Foster et al., 1996).

Sheep studies have previously recorded similarities between CH1641 and BSE. Foremost is the targeting of PrP genotypes of sheep following experimental challenge: both TSE isolates cause disease, with the shortest incubation periods being in QO111 sheep (Goldmann et al., 1994). The PrPSc glycoform patterns isolated from the brain of CH1641- and BSE-affected sheep (Hope et al., 1999) are also similar, although the two TSE sources differ markedly in mouse transmission characteristics. Here we have shown a further difference between these two isolates of TSEs.

In contrast to the icc results from SSBP/1, Western blotting indicated that PrPSc could be extracted from the brains of goats challenged both with SSBP/1 and with BSE. Although a direct comparison of PrP quantities from these protein blots may be misleading, it is evident that the intensity of staining of PrPSc is less for SSBP/1 than for BSE. The juxtaposing of results between positive PrPSc detection by Western blotting from SSBP/1 brain and the negative or nearly negative results following icc may reflect relative concentrations of protein detectable by the two methods. During tissue preparation for Western blots, protein is concentrated from substantially greater quantities of brain than those examined in icc sections. Although Western blot samples were taken from the thalamic area, some adjacent tissue may also have been included and the PrPSc fraction of the tissue was then concentrated. It is clear that the tissue sections used for icc have much less volume and so would be expected to contain very much less total PrPSc.

It is possible that immunoreactive epitopes on the PrPSc molecule may have remained undetected with icc. This could have occurred either because of the specific nature of the two monoclonal antibodies (FH11 and BG4) and possible variations in cleavage of the N terminus of PrPSc between SSBP/1 and BSE, or because some PrPSc epitopes remained masked, perhaps as a result of PrPSc protein structural conformation. The icc results achieved with FH11 and BG4 for known natural scrapie cases and the experimental BSE cases were similar and highly repeatable for distribution and intensity of PrPSc immunostaining. In an attempt to deal with this more fully, two polyclonal antisera (1A8 and 1B3) were used on a proportion of affected goats. The epitopes recognized by both 1A8 and 1B3 are extensive through much of the C-terminal part of the PrP molecule (see Methods); however, they revealed almost identical immunostaining to those of FH11 and BG4, confirming the former observations.

Taken together these results can be explained by PrPSc in goats generated from scrapie sources SSBP/1 and CH1641 having different conformations from that accumulating in BSE in goats. An association between the variation in PrPSc conformation and different infecting sources of TSE was recently demonstrated by Safar et al. (1998).

There are also differences in the pattern of protein banding, indicating differing levels of each of the three possible glycoforms of the PrPSc protein between SSBP/1 and BSE. SSBP/1 appeared to generate appreciably more of the unglycosylated form of PrPSc and less of the diglycosylated form than is apparent with BSE, even for preparations of non-proteinase K-treated PrPSc. Whether these differences in glycosylation patterns between SSBP/1 and BSE could have been responsible for the dissimilarities in PrPSc immunorecognition observed with icc is unknown.

The glycoform pattern has been suggested as a possible means of distinguishing sources of TSE (Collinge et al., 1996; Hope et al., 1999) and, although the protocol used for glycoform preparation here varied slightly to that used in the referenced studies, our results lend some support to that idea. However, it should be stated clearly that these differences in glycoform patterns between BSE and the experimental source SSBP/1 should not be taken as evidence of a test to distinguish between BSE and all natural scrapie. In fact a recent French study has shown that glycoform profiling of PrP from French natural scrapie cases produced very similar patterns to those from cattle with BSE and other experimental TSE cases in mice (Baron et al., 1999).

Previous studies in sheep subcutaneously inoculated with SSBP/1 have produced weak immunostaining of PrPSc and minimal or no vacuolar degeneration in the brain of terminally affected animals (Foster et al., 1996). The present study has also shown quite clearly that PrPSc in TSE-affected goats can be at least partially dissociated from intense vacuolar degeneration, depending on the source of infection and means of PrP identification. Altogether, this indicates that in some instances of terminal TSE disease neither vacuolation nor PrPSc detection need necessarily be demonstrable.

The correlation between the appearance of PrPSc in infected tissue and TSE infectivity has been a matter of discussion for a long time. With the transmission of scrapie to sheep by inoculation of homogenized brain taken from the thalamic area of one of our SSBP/1-infected goats we have demonstrated the presence of infectivity in an area of intense vacuolation but little detectable PrPSc. Furthermore, the incubation periods of these bioassay sheep suggested no evidence of a significant reduction in infectivity titre compared with published data of SSBP/1 transmission in sheep (Goldmann et al., 1996), considering that both PrP genotype and the species was changed in these transmissions.

Our results, therefore, emphasize the importance of using several techniques for an unambiguous diagnosis of TSE infection. Reliance on a single method may lead to the misinterpretation of results by missing genuine TSE cases.

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References


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