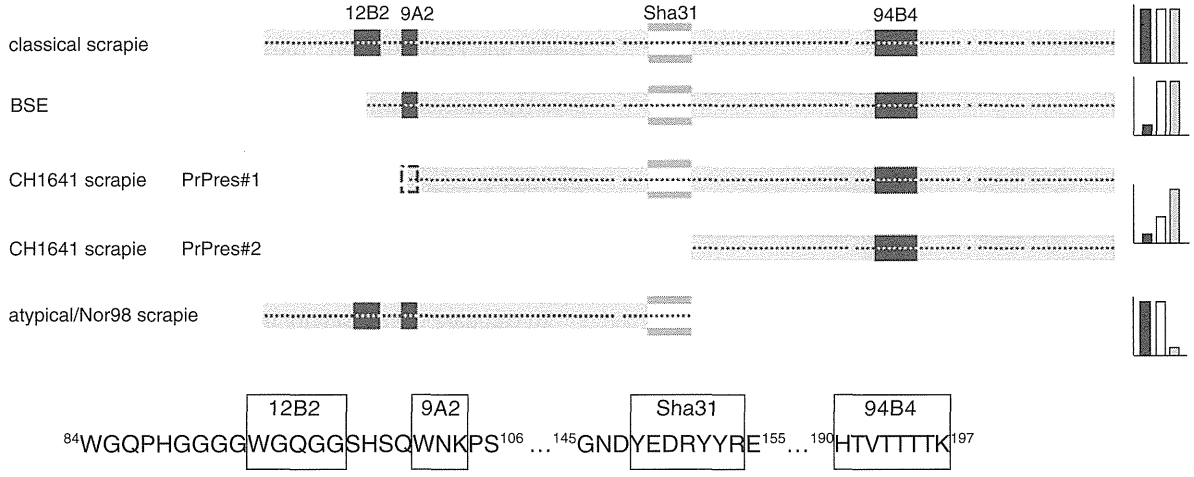

Fig. 1. The principle of discrimination of BSE/CH1641, scrapie and atypical scrapie using multiplex IFMA. After PK digestion, 9A2, 12B2 and 94B4 antibodies can all bind to classical scrapie PrP^{res}; only 9A2 and 94B4 bind substantially to BSE and CH1641 PrPres; only 12B2 and 9A2 bind substantially to atypical scrapie PrP^{res}. The capture antibodies 12B2 for amino acid residues 93–97, 9A2 for ovine PrP N-terminal amino acid residues 102–104, and 94B4 for amino acid residues 190–197, are coupled to distinct bead types. The reporter antibody Sha31 (epitope; residues 148–155) is biotinylated. Theoretical idealised profiles of MFI readings, associated with 12B2 (black filled bar), 9A2 (unfilled bar) and 94B4 (grey filled bar) respectively, are indicated on the right side of the simplified representation of the PrP^{res} protein primary structure. CH1641 may be differentiated from BSE by analysis of a minor 14 kDa C-terminal fragment and also, the 9A2 region might be different from BSE which would result in lower 9A2 values than in BSE (Jeffrey et al., 2006; Baron et al., 2008). Only PrPres#1 of CH1641 can be detected by mIFMA as the Sha31 binding epitope is missing in PrPres#2.

2.2. Tissue sample and preparation

Brain stem tissues (Table 1) were obtained from the Biological (TSE) Archive, Animal Health and Veterinary Laboratories Agency, UK, and from the archives in The Netherlands, France and Japan. Classical and atypical scrapie affected sheep and BSE affected cattle were naturally infected. BSE affected sheep were experimentally infected by oral inoculation with bovine BSE brain stem homogenate. Experimental CH1641 materials were all from sheep challenged intracerebrally either at AHVLA or two sheep and a goat challenged at the Lelystad facilities or one sheep challenged at the Tsukuba facilities in Japan (Yokoyama et al., 2010); these isolates are referred to simply as CH1641. The inoculum used for the Dutch three cases was obtained from Jim Foster (UEDIN-Roslin, Edinburgh) (Foster and Dickinson, 1988). Moreover, CH1641-like field case materials with highly reduced mAb P4 or 12B2 reactivity were from France from the archive of ANSES—Lyon (two cases, (Baron et al., 2008)) and from INRA-ENVT Toulouse. Control samples were confirmed as free of TSE infection and were obtained from sheep which were direct progeny of New Zealand stock (New Zealand is believed to be free from classical scrapie infection), and maintained and bred on a closed, secure farm, isolated from potential sources of TSE infection. Disease status, in all cases, was confirmed by neuropathological examination (brain pathology and immunohistochemistry) and WB analysis.

2.3. Proteinase K digestion and denaturation of PrP

Sample homogenates (10%) were prepared as described previously (Tang et al., 2010). Homogenate supernatants (100 µl) were digested at 50 °C for 1 h with 50 µg PK/ml homogenate (Merck, Darmstadt, Germany, specific activity 45.5 U/mg; a stock solution was prepared containing 11 mg PK/ml in 50 mM Tris pH 8.0 and 1 mM $CaCl_2$). The reactions were stopped by quantitative precipitation of PrP^{res} (Jacobs et al., 2007) with 1-propanol (110 µl) and centrifugation (16,000×*g* for 6 min; Jacobs et al., 2007). Pellets were dissolved in 12.5 µl TeSeE Reagent C (TeSeE™ sheep/goat Purification Kit, Bio-Rad) and denatured at 100 °C for 7 min. This denaturation process is critical to the assay as in all PrP^{Sc} targeted diagnostic methods (Tang et al., 2010). Finally, the denatured samples were diluted by adding PBS (42.5 µl) containing 1% BSA (PBSB) and centrifuged at 16,000×*g* for 6 min to remove insoluble debris and analysed directly by mIFMA. For analyses carried out independently by mass spectrometry, digestion with PK (100 µg/ml homogenate) was carried out at 50 °C for 60 min in Prionics-check WESTERN Homogenisation Buffer (Prionics, Swizerland) as described (Gielbert et al., 2009).

2.4. mIFMA

Coupling of capture antibodies to assay beads (Bio-Plex™ COOH beads, Bio-Rad, Hemel Hempstead, Herts, UK) and biotinylation of reporter antibody Sha31 antibody (EZ-Link Micro NHS-PEO_4-Biotinylation Kit, Pierce, Rockford, IL, USA) were according to the manufacturer's instructions and as described previously (Tang et al., 2010).

The IFMA here used was a triplex assay and was essentially as described previously for the simultaneous incubation diplex IFMA using mAbs 9A2 and 12B2 (Tang et al., 2010) but with inclusion of an additional capture mAb (94B4). Briefly, a mixture of capture antibody beads 12B2, 9A2 and 94B4 (5000 of each bead type), biotin labelled Sha31 as a reporter (2.43 µg/ml) and streptavidin-R-phycoerythrin (2 µg/ml) was prepared in PBSB buffer on the day of use. The mixture (50 µl) was added to PK treated sample supernatants (50 µl) contained in 96 well filtration plates and incubated with shaking at room

Table 1
Brainstem tissue samples.

Sample number	Animal ID	Genotype	PrP^{res} by WB
Ovine negative			
1	PG 437/06	ARQ/VRQ	–
2	PG 438/06	ARQ/VRQ	–
3	PG 1107/05	ARQ/VRQ	–
4	PG 461/03	ARQ/VRQ	–
5	PG 1700/05	VRQ/VRQ	–
Ovine classic scrapie			
6	PG 1251/05	ARQ/VRQ	++
7	PG 402/04	ARQ/ARQ	+++
8	PG 1850/02	VRQ/VRQ	++
9	PG 280/04	ARQ/VRQ	+++
10	PG 1276/02	VRQ/VRQ	+
11	PG 964/03	ARQ/ARQ	+++
39	CVI 398	ARQ/VRQ	+++
Ovine experimental scrapie			
52	NIAH 73	ARQ/VRQ	+++
Ovine experimental BSE			
12	PG 167/04	ARQ/ARQ	++
13	PG 389/04	ARQ/ARQ	++
14	PG 305/04	ARQ/ARQ	+++
15	PG 0473/04	ARQ/ARQ	+++
16	PG 0392/04	ARQ/ARQ	++
Ovine atypical scrapie			
17	SS4-040875	ARR/AHQ	++
18	SS4-037510	ARR/AHQ	++
19	SS4-035907	AHQ/AHQ	++
20	SS4-038223	ARR/ARR	++
21	SS0595526	ARR/ARQ	++
22	PG0108/09	AHQ/AHQ	+
23	PG0497/07	AHQ/AHQ	++
24	PG1270/06	ARR/AHQ	++
25	PG0832/06	ARQ/ARQ	++
26	PG0076/06	ARQ/ARQ	++
27	PG0667/06	ARR/ARQ	+
40	CVI 433	AHQ/ARQ	++
Ovine experimental CH1641			
28	PG1276/05	AHQ/AHQ	++
29	PG1271/05	AHQ/AHQ	++
30	PG0851/05	AHQ/AHQ	++
31	PG1283/05	AHQ/AHQ	++
32	PG1275-05	AHQ/AHQ	++
33	PG1284-05	AHQ/AHQ	++
41	CVI 8015470	ARQ/ARQ	++
42	CVI 8015471	ARQ/ARQ	++
44	NIAH 56	ARQ/VRQ	++
Caprine experimental CH1641			
43	CVI 8018337	WT goat	++
Ovine natural CH1641-like isolate			
45	ANSES 06-0287	ARQ/ARQ	++
46	ANSES 06-0412	ARQ/ARQ	+++
47	INRA PS048	VRQ/VRQ	++
48	INRA PS129	VRQ/VRQ	+
49	INRA PS165	VRQ/VRQ	++
50	INRA PS227	VRQ/VRQ	+
51	INRA PS303	VRQ/VRQ	++
Bovine negative			
34	PG 139/00		–
35	PG 0227/03		–
36	PG 2054/00		–
Bovine BSE			
37	RBSE 01/683		++
38	RBSE 01/114		++

PrP^{res} signal strength: +++, strong; ++, medium; +, weak; –, none. WB: Western blotting analysis with 6H4 and P4 antibodies (Stack et al., 2002). Sample 1–38: AHVLA, UK; Sample 39–43: CVI, The Netherlands; Sample 44 and 52: MIAH, Japan; Sample 45–46: ANSES, France; and Sample 47–51: INRA-ENVT, France. Sample 43 is from an experimentally infected wt PrP goat. Experimental CH1641 samples 28–33 and 41–43 were infected with the same CH1641 material supplied by UEDIN-Roslin, Edinburgh, UK.

temperature for 90 min before washing twice by sequential filtration and PBSB addition (100 µl). Washed beads were suspended in PBSB (100 µl) and PrP binding parameters for each bead type analysed using a dedicated multiplex flow cytometry based instrument and associated software (Bio-plex™200 system and Bio-Plex Manager version 4, Bio-Rad). Values are reported as median fluorescence intensity (MFI) units.

2.5. Western Blotting analysis

To compare the sensitivity of IFMA and western blotting analysis, atypical scrapie infected brain tissue samples were analysed with the TeSeE Universal WB (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions. In brief, tissue samples were ribolysed, purified, PK treated and denatured. Each sample was loaded in single lanes onto pre-cast 12% bis-tris gels (Criterion, Bio-Rad) and electrophoresed for 50 min at 200 V. The proteins were then transferred onto PVDF membranes (115 V for 60 min) and blocked (Bio-Rad blocking solution) for 40 min at room temperature. Blots were probed with the kit primary antibody for 30 min at room temperature. The membranes were washed, incubated for 20 min in Bio-Rad secondary antibody-horse radish peroxidase conjugate at room temperature, washed again and the membranes incubated with ECL substrate (GE Healthcare, Amersham, UK) for 45–60 s. The images were digitally recorded using a Fluor-S MultiImager (Bio-Rad). For the purpose of these studies, blots were imaged following exposure for 10 min (enhanced images) to ensure that samples containing comparatively low PrP^{Sc} concentrations were detectable.

2.6. Quantification of N-terminal PK cleavage site by mass spectrometry

The quantities of PrP^{res} associated with individual PrP^{Sc} PK cleavage sites were determined by N-terminal amino acid profiling (N-TAAP, based on the principle described previously; Gielbert et al., 2009). This enabled the quantification of PrP^{res} associated with the epitopes of 12B2, 9A2 and 94B4 from CH1641 and BSE. Briefly, all the possible N-terminal tryptic peptides of PrP^{res} (in the sequence between G77 and W102, and which have the C-terminal amino acid residue K109) were first investigated and identified by accurate mass measurement (Chip-HPLC/MS Q-Tof 6520, Agilent, Reading, UK) for classical scrapie, ovine BSE and CH1641 scrapie (Fig. S1). Identified peptides were synthesised to provide calibration standards for quantification by multiple selected reaction monitoring (mSRM). Brain homogenate (equivalent to 75 µg tissue) was digested first with PK and then (under denaturing conditions) with trypsin. The resulting peptides were quantitated following injection of an aliquot (equivalent 1 µg tissue) of the peptides generated and were injected into a 6410 triple quadrupole mass spectrometer using a 1200 nanoHPLC system (Agilent) and a Chipcube interface. The data were analysed using proprietary software (MassHunter Quantitative Analysis software, Agilent). A range of concentrations of calibration standards were run for each peptide within each sample batch. The concentration of each peptide in the samples was calculated from their respective

ion counts (Fig. S2) by interpolation from the peptide calibration curves.

2.7. Statistical analysis

Student's *t*-test was used to compare the data from TSE samples with those from the controls (two tailed distribution for two-sample populations of unequal variance) using a standard spreadsheet function (Microsoft Excel). P values ≤ 0.05 were regarded as significant.

3. Results

The aim of these studies was to develop a simple multiplex assay with the capability to differentiate simultaneously the major TSE types in sheep: classical scrapie, atypical scrapie, CH1641 scrapie and ovine and bovine BSE. The triplex IFMA developed and applied here was based on the previously described duplex assay (Tang et al., 2010), incorporating a third mAb (94B4) with specificity for a core PrP residue sequence (Fig. S1). This was to enable additional differences in PK truncation associated with atypical scrapie PrP^{Sc} compared with classical scrapie and BSE to be distinguished. There is substantial evidence, previously identified by WB analysis (Gretzschel et al., 2006; Klingeborn et al., 2006), that there is significant truncation of atypical scrapie PrP^{res} in the C-terminal domain compared to PrP^{res} of BSE and classical scrapie and further that the N-terminus is less truncated than BSE PrP^{res} (Fig. 1). In the studies described here, mIFMA enabled identification and semi-quantitation of differences in truncation by employing N-terminal capture of PrP^{res} by mAbs 12B2 and 9A2 and C-terminal capture by mAb 94B4. This was combined with the biotinylated reporter mAb Sha31 and streptavidin-R-phycoerythrin to determine the binding endpoint. This choice was hypothesised to lead to unique binding profiles, which are specific and differentially diagnostic for classical scrapie, atypical scrapie, BSE and CH1641 (Fig. 1).

3.1. Antibodies 12B2 and 94B4 are sufficient differentiation for scrapie, BSE and atypical scrapie

Differentiation of classical scrapie, BSE and atypical scrapie required employment of only two of the three capture antibodies, namely 12B2 and 94B4 (Fig. 2). Both these antibodies bind PrP^{res} of classical scrapie, as determined using the reporter antibody, Sha31. However, since atypical scrapie PrP^{res} has a truncated C-terminal end that does not bind 94B4, only the region binding 12B2 is reported by IFMA. The degree of N-terminal truncation of BSE PrP^{res} dictates that only 94B4 binding is reported substantially, since the 12B2 epitope is largely cleaved by PK digestion. Comprehensive PK digestion of PrP^{C} in the negative control samples was evident from the low baseline MFI value obtained (Fig. 2). For classical scrapie, essentially all the capture antibody binding sites remain intact and were detected with the reporter antibody. Assuming equal distribution of binding for both 12B2 and 94B4, a binding ratio of ~1 was anticipated and indeed was found (Fig. 2). Similarly, binding ratios of ~1 were observed for 9A2 antibody binding (Tang et al., 2010). By the same principle, predicted 12B2/94B4 binding ratios were <1 for BSE and >1 for atypical scrapie (Fig. 1), which was also in keeping with the test outcomes (Fig. 2).

The assay fluorescence end-point for negative control samples was similar to that for PBS control sample ($n=6$, typically around 300–2000 MFI units and distinct from the specific signal range associated with PrP^{res} binding of the fifteen TSE samples; Fig. 2). For the TSE samples, binding to either 12B2 or 94B4 (whichever is the highest, dependent on TSE type) was in the range 18,000–29,000 MFI units, suggesting a high predictive value for TSE diagnosis. To illustrate a worst case situation for assay performance, all test sample outcomes for both capture antibodies (all mean MFI values, Fig. 2) were taken together as a single population. Analysis of differences between test ($n=15$) and control samples ($n=6$) indicated a highly significant difference ($p=1.88\times10^{-6}$, *t*-test, assuming equal variance).

Binding to 94B4 of PrP^{res} from BSE samples was of similar order (>18,000–28,300 < MFI units) to that for classical scrapie (Fig. 2) but was near background levels for 12B2 antibody (>1200–5500 < MFI units). Association of atypical scrapie PrP^{res} with 12B2 was also substantial (>28,000–34,400 < MFI units) and, in keeping with the model (Fig. 1), low for 94B4 antibody (>1500–2100 < MFI units).

The differences between 12B2/94B4 binding ratios for classical scrapie (range 0.82 to 1.11, $n=6$), BSE (range 0.02 to 0.2, $n=4$) and atypical scrapie (range 14.3 to 21, $n=5$) were highly significant (classical scrapie versus BSE: $p=3.29\times10^{-7}$; classical scrapie vs atypical scrapie: $p=1.21\times10^{-4}$; BSE vs atypical scrapie: $p=9.84\times10^{-5}$) and indicative of the strong capability of mIFMA to differentiate TSE strains (Fig. 2).

3.2. mIFMA detects atypical scrapie with sensitivity comparable to Western blotting analysis

For all five atypical scrapie samples available to this study, 12B2 binding of PrP^{res} approached saturation level, as indicated by the near upper limit fluorescence value obtained for each (~30,000 MFI units). This suggests that mIFMA provides a highly sensitive test for atypical scrapie even under conditions of high PK concentration and high temperature conditions typically used, as here, for BSE and scrapie sample analysis. The sensitivity potential of mIFMA was compared with WB (Arsac et al., 2007) by analysing a dilution series of six atypical samples (Fig. 3; WB images shown were generated following an extended exposure time to allow visualisation of samples with low PrP^{res} content). Standard tissue sample quantities were 10 and 19 mg for the methods used in mIFMA and WB analysis, respectively. Findings indicated that the PrP^{Sc} concentration was low in sample 22 as the undiluted sample homogenates gave significantly higher signals than the background and the 12B2 signal was only marginally higher than the background when 0.5 mg of the sample was used in the assay (20 fold dilution, Fig. 3B). For WB analysis, the PrP^{res} content fell effectively below background at 0.38 mg tissue per well. Thus there was a good correlation between the assays, mIFMA giving at least equivalent sensitivity as WB analysis (Fig. 3). It is worth noting that in all cases 12B2 mAb provided the most sensitive detection of PrP^{res} compared with 9A2, since binding for 9A2 rapidly reduced to background levels with increased sample dilution

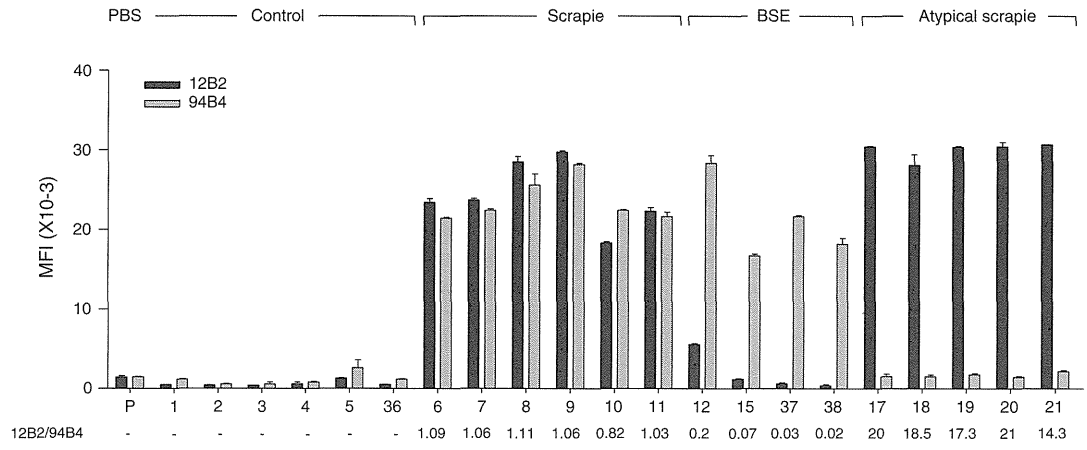

Fig. 2. mIFMA applied to the detection and discrimination of atypical scrapie, ovine BSE and classical scrapie. Sample homogenates (~0.2 mg brain equivalent at 50 fold dilution of original homogenate) from individual sheep were analysed in replicate (n = 2). Values represent mean MFI ± range of duplicate determinations. The identity of individual samples is given in Table 1. P = PBS buffer blank.

which is probably due to the superior affinity of mAb 12B2 compared to the other capture mAbs (Fig. 3B).

3.3. CH1641 PrP^{res} is less abundant than BSE PrP^{res} in the region of the mAb 9A2 epitope

Published data to date (Stack et al., 2002; Baron et al., 2008; Jacobs et al., 2011) indicate that PrP^{Sc} from BSE and CH1641 is similarly truncated by PK and as a consequence, it has proved challenging to distinguish the respective PrP^{res} products using biochemical methods based on antibody binding characteristics. However, Baron et al. have identified a 14 kDa C-terminus fragment associated with CH1641 PrP^{Sc} cleavage which is not evident from the equivalent BSE PrP^{Sc} products as determined by WB analysis (Vulin et al., 2011). When BSE samples (n = 4) were compared with experimental

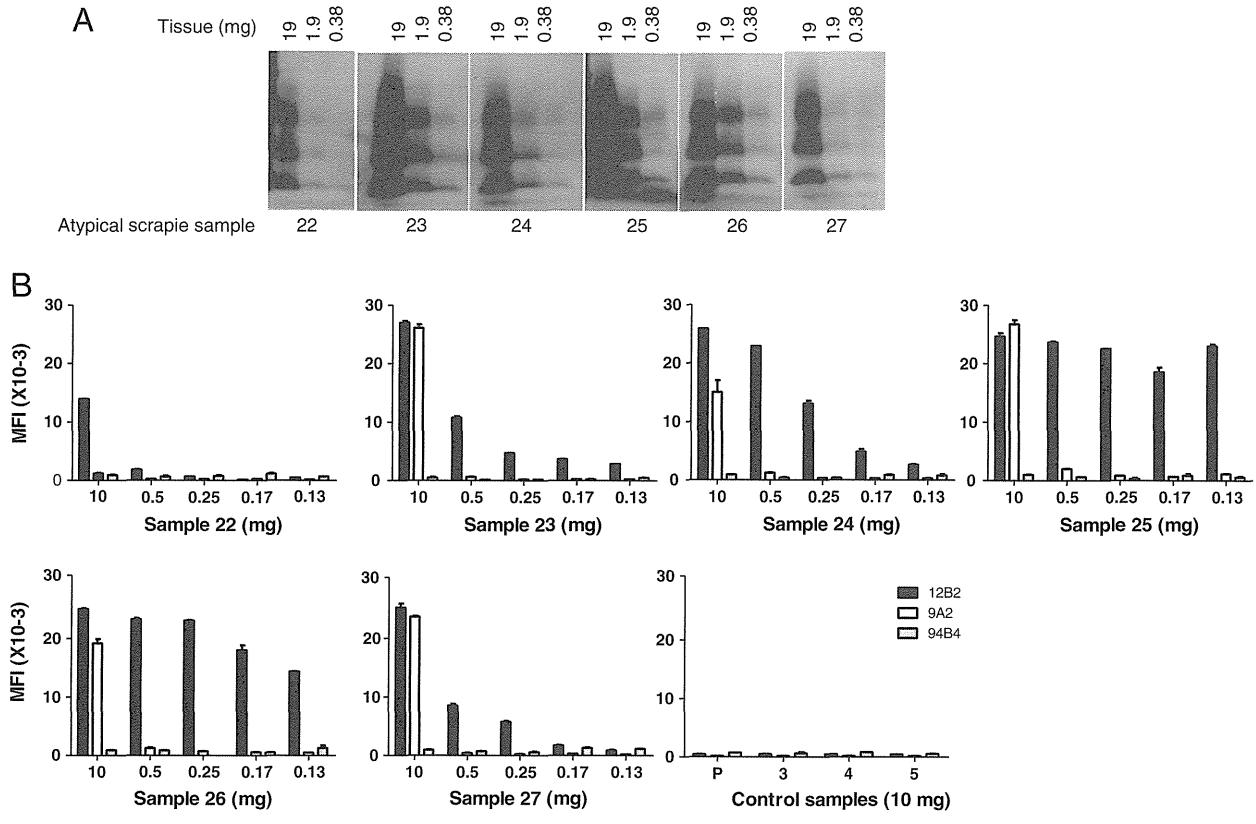

Fig. 3. A comparison of the detection sensitivity of atypical scrapie by Western blotting analysis and mIFMA following serial sample dilutions. Panel A, WB analysis with the antibody P4. Panel B, mIFMA with the antibodies 12B2, 9A2 and 94B4. Values represent mean MFI ± range of duplicate determinations. The identity of individual samples is given in Table 1. P = PBS buffer blank.

CH1641 (n = 10) samples and natural CH1641-like (n = 7) isolates (Table 1), using mIFMA, a highly significant difference was observed between binding ratios of 9A2 and 94B4 both for experimental CH1641 ($p = 9.02 \times 10^{-5}$, *t*-test, equal variance) and the CH1641-like field cases ($p = 1.24 \times 10^{-3}$, *t*-test, equal variance, Fig. 4). The ratios for BSE were between 0.91 and 1.03 and for CH1641 between 0.03 and 0.63 (Fig. 4). This indicated that the 9A2/94B4 ratio provides a strong basis for discrimination of BSE and CH1641. Thus, the triplex mIFMA was able to differentiate readily all four prion disease variants investigated: classical scrapie, atypical scrapie, CH1641 scrapie and BSE.

More fundamentally, the reduced 9A2 binding values suggest that cleavage of the N-terminus extends further towards the C-terminus for CH1641 than is the case for BSE (Fig. 4) since the mean MFI units associated with 9A2 for BSE (19.4 ± 2.2 sd) were 2.06 fold lower than in experimental CH1641 (9.4 ± 4.8 sd; $p = 5.86 \times 10^{-42}$, *t*-test, equal variance), and 2.13 fold lower than in CH1641-like isolates (9.14 ± 6.19; p = 0.011, *t*-test, equal variance).

It is interesting to note that binding associated with 9A2 varied appreciably between all CH1641 samples regardless of whether they were derived experimentally , or from natural sources, suggesting that the PrP^{res} structure in the 9A2 region differs slightly between the isolates. The ratios of 12B2/94B4 and 9A2/94B4 for CH1641-like samples 47–51 were essentially indistinguishable from those of the experimental CH1641 samples (Fig. 4).

To provide an independent assessment of the quantities of PrP^{res} types associated with binding of 12B2, 9A2 and 94B4 antibodies, PrP^{res} preparations from CH1641 and BSE affected sheep (n = 2 animals for each) were analysed by mSRM mass spectrometry. The PrP peptides derived from PK treatment of the samples followed by tryptic digestion under denaturing conditions (see Materials and methods) were quantified by interpolation from calibration curves derived using dilutions of individual peptide standards. PK digestion of PrP^{Sc} gives rise to multiple products which comprise PrP^{res} and thus, following tryptic digestion, each of the mAb binding domains is represented by a number of peptides, the sum of their concentrations providing an estimate of PrP^{res} associated with each domain (Fig. S1). Thus 12B2 is represented by the sum of eight peptides in region from G77 to K109 whereas 9A2 is represented by twelve peptides within the region G77 to K109. A single tryptic peptide (Q189 to K197) represents the 94B4 binding domain. In the 9A2 binding region, PrP^{res} was 2.2 fold less abundant (*t*-test; p = 0.043) in CH1641 than in BSE (Fig. 5), consistent with the difference in binding observed by mIFMA. When the same assay was carried out using more stringent PK digestion conditions with six BSE and six experimental CH1641 samples, the same conclusions were drawn as only the 9A2 region was reduced in CH1641 compared with BSE (data not shown).

4. Discussion

Application of the precautionary principle to TSE surveillance in livestock remains appropriate, given uncertainties about the possibility, however unlikely, that the agent responsible for BSE in cattle may have entered the sheep population through contaminated feed. Development and application of assays which are cost effective, in proportion to risk and which are able to both screen samples and characterise type dependent variations are thus part of surveillance strategies to minimise the risk to the consumer of BSE

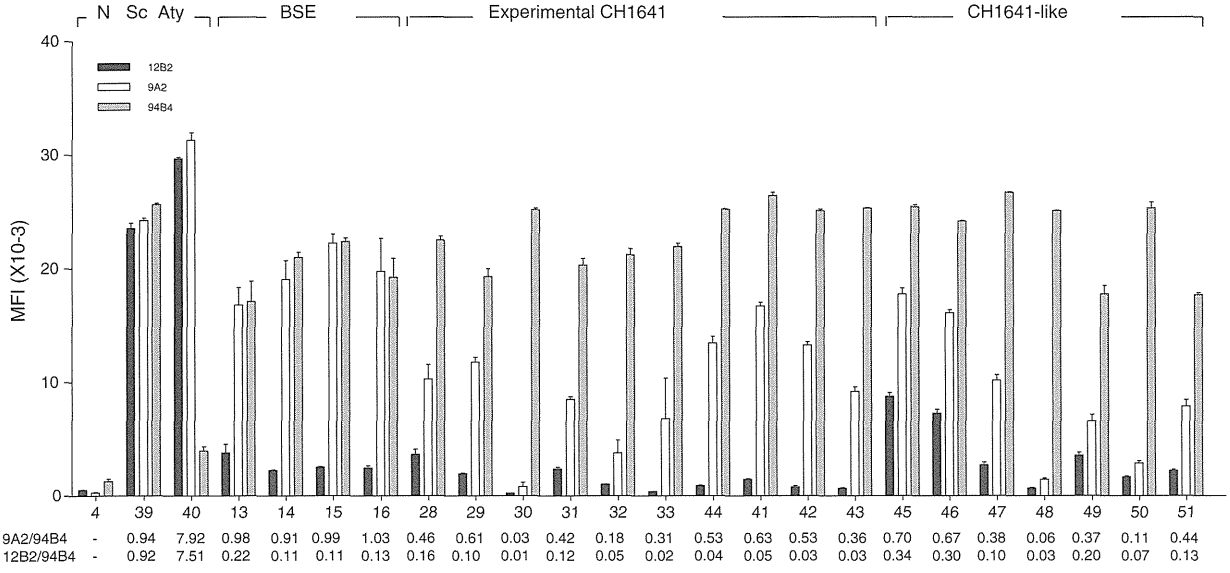

Fig. 4. Differentiation of BSE and CH1641. Sheep brain stem samples from experimental CH1641 and natural isolates of CH1641-like and BSE affected cases show distinct differences in mIFMA binding ratios between 9A2 and 94B4. Sample homogenates from individual sheep (negative (N), n = 1; classical scrapie (Sc), n = 1; atypical scrapie (Aty), n = 1; experimental CH164, n = 10; CH1641-like field cases, n = 7; experimental BSE, n = 4) were analysed in duplicate (n = 2). Values represent mean MFI ± range. The ratio of MFI values between 9A2 and 94B4 and between 12B2 and 94B4 is indicated for each TSE sample. The identity of individual samples is given in Table 1.

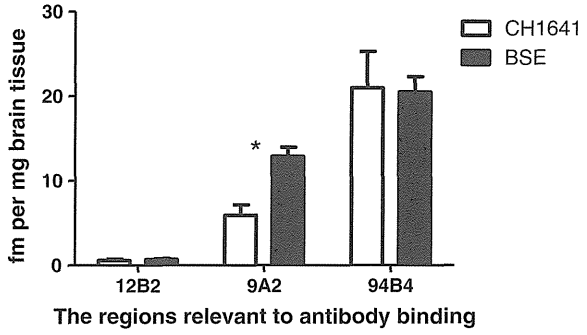

Fig. 5. The absolute quantity of peptides in PrP^{res} associated with capture antibody binding domains by mSRM analysis. The total quantities of PrP^{res} peptides associated with the epitopes of 12B2, 9A2 and 94B4 were measured in duplicate and compared between CH1641 (n = 2) and BSE (n = 2). The 12B2 region contains eight peptide fragments covering amino acid residues 77 to 95 (Fig. S1). The 9A2 region contains twelve peptide fragments covering residues 77 to 102. The 94B4 region contains the tryptic peptide fragment from residue 189 to 197. *: $p<0.05$.

entering the food chain. This is important because of the zoonotic association between BSE in cattle and variant Creutzfeldt–Jakob disease in humans.

A simple diplex IFMA enabling differential diagnosis of BSE and scrapie in sheep has been described previously (Tang et al., 2010). In the present study, this approach was further developed by investigation of different antibody combinations and use of a triplex format to enable clear cut discrimination of atypical scrapie concurrent with classical scrapie and BSE. Test data support strongly the possibility that the triplex IFMA can further distinguish unusual forms of scrapie with a BSE-like molecular profile (e.g. CH1641) from BSE. It was found that analysis of PrP^{res} binding of the three capture mAbs 12B2 and 94B4 by mIFMA was sufficient to distinguish negative controls, classical scrapie, BSE and atypical scrapie.

The limited data presented here suggest that mIFMA provides sensitive detection of atypical scrapie, comparable to Western blotting. Furthermore, the widely appreciated requirement for adjustment of assay conditions (lower temperature, shorter incubation times or lower concentrations of PK), to take account of implied increased sensitivity of PrP^{Sc} to PK digestion (Buschmann et al., 2004; Everest et al., 2006; Klingeborn et al., 2006), was not necessary for mIFMA. The PrP^{res} sequences targeted provided for application of the same simple format and conditions to all TSE types: all five atypical scrapie samples tested by mIFMA gave consistently high MFI readings.

Use of the 12B2, 9A2 and 94B4 capture antibody triplex provided additional differentiation of BSE from CH1641. This represents an important advance in diagnostic capability since this facilitates the ability to investigate unusual scrapie types such as CH1641 within the sheep population alongside better characterised TSE isolates. Such unusual isolates have presented a challenge for biochemical differential diagnosis because the corresponding PrP^{res} shares some of the molecular features with BSE PrP^{res}. To date routine biochemical differential tests have been largely reliant on WB analysis but banding patterns for BSE and CH1641-like isolates have until recently remained indistinguishable. In a recent study, it was observed that a small proportion of PrP^{res} from transgenic mouse passaged CH1641 was represented by a 14 kDa C terminal region product, which was not evident in BSE PrP^{res}. This provided the possibility of biochemical differentiation of BSE and CH1641-like scrapie (Baron et al., 2008). The profiles between experimental CH1641 and CH1641-like were similar in mIFMA, suggesting that the ratio between 9A2 and 94B4 should essentially be linked in WB to the PrP^{res}#1 population also often referred as the 19 kDa fragment (Baron et al., 2008). Two of seven CH1641-like, samples 45 and 46, displayed elevated ratios of both 12B2/94B4 and 9A2/94B4, suggesting a slightly more PK resistant structure in the N terminus than that of BSE, experimental CH1641 and the other five CH1641-like cases (Fig. 4). Profiles may be the result of natural variation among CH1641-like isolates. Another explanation for the elevated ratios is that in field cases there could be two forms of PrP^{res} in the samples due to a mixed infection with classical scrapie PrP^{Sc} and CH1641 PrP^{Sc}. It has been shown that when sheep were inoculated with a USA scrapie field isolate, the animals developed experimental CH1641 (sample 44) and classical scrapie (sample 52) (Yokoyama et al., 2010). The ratio between two strains might depend on individual and flock TSE status. Clearly, more tests are needed to confirm these findings.

The reduced abundance of the PrP^{res} 9A2 epitope found here for both experimental CH1641 and CH1641-like cases was unexpected, especially given the extent of the difference (2.1 fold for both) compared with BSE. It was reasoned that since mAb 9A2 binding is relatively sensitive to small changes in PrP concentration (i.e. sample dilution gives substantially reduced binding to 9A2 compared to 12B2) both in classical scrapie (Tang et al., 2010) and atypical scrapie (Fig. 3B), a small reduction of CH1641 PrP^{res} concentration would result in the enhanced difference observed compared with BSE because of the relatively weak binding of 9A2. To test this hypothesis, more detailed analysis was carried out using mass spectrometry to fully quantify the constituents of PrP^{res} bearing this epitope. This analysis provided strong corroboration that the 9A2 epitope is less abundant in CH1641 PrP^{res} relative to BSE. This is consistent with the results of the immunohistochemical peptide mapping which shows that PrP^{Sc} from the CH1641 isolate was truncated further upstream in the N terminus than was PrP^{Sc} from ovine BSE (Jeffrey et al., 2006). In the present study the CH1641 samples were from both experimentally and naturally infected sheep. Thus mIFMA may prove to provide a more simple and robust approach for discrimination of CH1641 and experimental ovine BSE. Furthermore CH1641-like field case samples gave mAb binding profiles which were surprisingly similar to those from experimental CH1641 isolates, indicating that mIFMA distinguishes natural CH1641-like isolates from BSE as well as from classical scrapie.

In previous mass spectrometry based studies, in which N-terminal PK cleavage sites alone were compared for CH1641 and BSE, no obvious difference was evident between the BSE and experimental CH1641 (Gielbert et al., 2009). Increasing the sequence coverage of the prion protein with respect to the number of peptides quantified (addition of N-terminal PK cleavage sites S100 and W102, and of core- and C-terminal tryptic peptides, including Q189-K197, the latter covering the 94B4 epitope), however, enabled clear distinction between CH1641 and BSE when comparing the

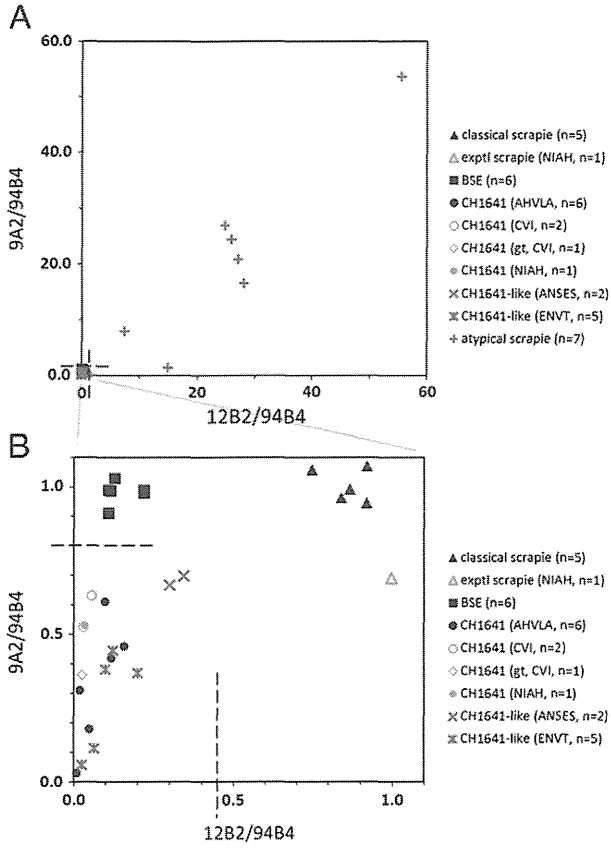

Fig.6. Dotplot overview of mIFMA derived mAb ratios for recognition of four TSE types in sheep, including the field cases with CH1641-like properties. The 12B2/94B4 and 9A2/94B4 ratios were determined in one single assay run. Panels A and B have the same samples. A: the ratio axes plotted from 0 to 60 displaying the isolated position of atypical case(s); B: the ratio axes plotted from 0 to 1.1. The dashed lines indicate distinct clusters of the three TSE types: CH1641-like field cases and experimental CH1641 cases from BSE cases and from classical scrapie cases.

respective ratios of the total N-terminal peptides to the core signal (Q189-K197).

The differentiation of classical scrapie, BSE, atypical scrapie and CH1641 can also be displayed comprehensively using dotplots, with 12B2/94B4 and 9A2/94B4 (Fig. 6). All TSE types were separated. Especially, all CH1641 samples (experimental and natural) with values below 0.8 and 0.5 respectively for 12B2/94B4 and 9A2/94B4 (area within the dashed lines of Fig. 6B) while atypical scrapie showed relatively distinct and high ratios (Fig. 6A). Thus, this mIFMA methodology appears suitable to discriminate the four TSEs in sheep and potentially also in goats: classical scrapie, CH1641-like scrapie, BSE and Nor98/atypical scrapie (Fig. 6). This ranks the mIFMA approach as a novel biochemical diagnostic technique for tissue homogenates suitable for both rapid screening as well as discriminatory and confirmative purposes.

More recent developments using multiplex WB glycoprofiling procedures have demonstrated differences in glycosylation state of PrP^{res} enabling CH1641 and CH1641-like isolates to be distinguished from BSE (Jacobs et al., 2011) and the differentiation of scrapie, BSE and CH1641-like can also be achieved using WB by combining the GdnHCl pre-treatment of brain homogenates and N-terminal cleavage by PK (Pirisinu et al., 2011). The possibility of including glycan capture antibodies within the mIFMA is a realistic option which may further extend the diagnostic power of this approach (Xanthopoulos et al., 2009).

In conclusion, this novel triplex approach enabled simultaneous discrimination of all four of the established TSE types known to be present in livestock. This suggests suitability of mIFMA for both screening and confirmatory differential diagnosis. The discovery of a reduction of both experimental CH1641 and CH1641-like PrP^{res} in the 9A2 binding region is a novel finding and may simplify diagnostic procedures for differentiation between classical scrapie, Nor98/atypical scrapie, CH1641 scrapie and BSE.

Supplementary data related to this article can be found online at doi:10.1016/j.jim.2012.03.004.

Acknowledgements

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12B2	9A2	94B4
G77-K109	G77-K109	
G81-K109	G81-K109	
G85-K109	G85-K109	
G89-K109	G89-K109	
G90-K109	G90-K109	
G91-K109	G91-K109	
G92-K109	G92-K109	
W93-K109	W93-K109	
	G94-K109	
	G96-K109	
	S100-K109	
	W102-K109	
		Q189-K197

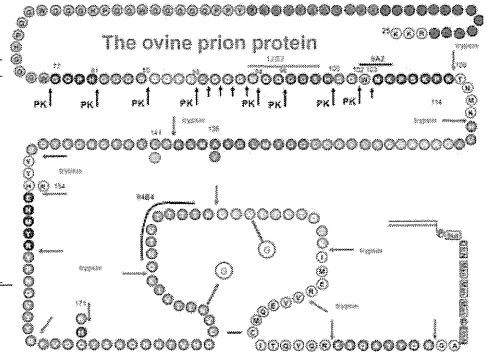

Figure S1. The peptides used to quantify PrP^{res} in the antibody binding regions are shown in the table. Eight N-terminally different peptides containing the 12B2 epitope and twelve containing the 9A2 epitope were identified using full scan (Q-Tof) mass spectrometry and specifically detected and quantified using triple quadrupole mass spectrometry. A single tryptic peptide contains the 94B4 epitope. The map of PrP provides the locations of mAB binding as well as showing the PK and tryptic cleavages resulting in the peptides listed in the table.

Ovine PrP tryptic and semi-tryptic peptide standards, 100 fmol on UHC chip

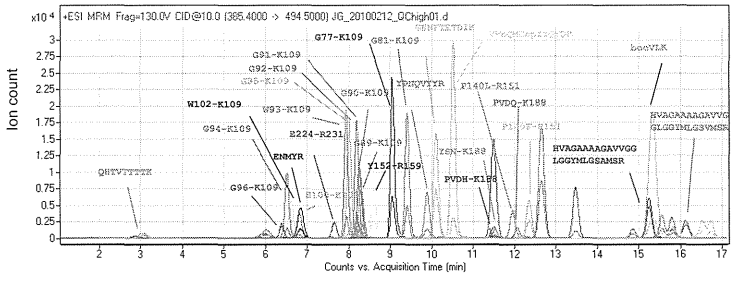

Figure S2. Chromatogram of LC-MS analysis of the standard peptide set when 1 fm of each standard peptide was added. The ion counts were compared to provide the quantity of a given peptide in the sample.

SHORT COMMUNICATION

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Comparative analysis of Japanese and foreign L-type BSE prions

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Key words: prion, atypical BSE, L-type BSE

Abbreviations: BSE, bovine spongiform encephalopathy; BSE/JP8, the 8th BSE case in Japan; BSE/JP24, the 24th BSE case in Japan; BASE, bovine amyloid spongiform encephalopathy; CNS, central nervous system; C-BSE, classical BSE; H-type BSE, high-type BSE; L-type BSE, low-type BSE; mAb, monoclonal antibody; PET blot, paraffin-embedded tissue blot; PK, proteinase K; PNGaseF, N-glycosidase F; PrP, prion protein; PrPcore, proteinase K-digested PrP^{Sc}; PrP^{Sc}, abnormal prion protein; TSEs, transmissible spongiform encephalopathies

L-type bovine spongiform encephalopathy (BSE) is an atypical form of BSE. To characterize the Japanese L-type BSE prion, we conducted a comparative study of the Japanese and foreign L-type BSE isolates. The L-type BSE isolates of Japan, Germany, France and Canada were intracerebrally inoculated into bovinized prion protein-overexpressing transgenic mice (TgBoPrP). All the examined L-type BSE isolates were transmitted to TgBoPrP mice, and no clear differences were observed in their biological and biochemical properties. Here, we present evidence that the Japanese and Canadian L-type BSE prions are identical to those from the European cases.

Bovine spongiform encephalopathy (BSE) is one of the transmissible spongiform encephalopathies (TSEs), or prion diseases, in cattle. TSE is characterized by spongiform changes in the central nervous system (CNS) and the accumulation of an abnormal prion protein (PrP^{Sc}) in the CNS.[1] PrP^{Sc} has been regarded as the major component of TSE pathogens.[2]

BSE was detected in the UK in 1986,[3] and subsequently spread to the other European countries, Japan and North America.[4-6] BSE is thought to be caused by a single prion strain, based on the analyses of its biological and biochemical characteristics.[7] From 2003, however, several atypical neuropathological and molecular phenotypes of BSE (atypical BSE) have been detected in Japan, several European countries and North America.[6,8-17] Currently, based on the molecular size of the proteinase-digested non-glycosylated form of PrP^{Sc}, atypical BSE is classified into two groups (L-type and H-type).[14]

L-type BSE cases have been identified in the European countries, including Italy, France, Germany, Netherland, Poland and in Canada and Japan.[8-15] Two L-type BSE cases have been identified in Japan. One case was detected in a healthy 23-mo-old Holstein steer (BSE/JP8),[8] and the other was detected in a 14-y-old black Japanese beef cattle (BSE/JP24).[9] The latter case was successfully transmitted to bovinized transgenic mice and cattle, and the biological and biochemical properties differed from that of classical BSE (C-BSE).[18,19] However, it is unclear whether Japanese L-type BSE prion is identical to that of L-type BSE isolates from other countries. To characterize the Japanese L-type BSE isolate, we performed a comparative study of the Japanese and foreign L-type BSE isolates.

A transmission study using experimental animals is a useful approach for prion characterization. Therefore, we performed a transmission study of the L-type BSE isolates in bovinized prion protein (PrP)-overexpressing transgenic mice (TgBoPrP).[20] Brain samples of L-type BSE-affected cattle from Japan (BSE/JP24),[9] France,[10] Germany[11] and Canada[12] were used in this study. The brain homogenates were intracerebrally inoculated into TgBoPrP using previously described methods in reference 18. All animal experiments were reviewed by the Committee of the Ethics on Animal Experiment of the National Institute of Animal Health.

All the examined L-type BSE isolates were transmitted to TgBoPrP, and the affected mice developed progressive neurological diseases. Japanese L-type BSE isolate-affected TgBoPrP exhibited a unique clinical sign, the circling behavior. The same phenotype was observed when TgBoPrP were inoculated with German, French and Canadian L-type BSE isolates. On the other hand, in the first passage the incubation period for the Japanese L-type BSE isolate was significantly different from that of the other L-type BSE isolates (**Table 1**). We then performed serial passages of these isolates for further characterization. The incubation periods in the second passage were shorter than those in the first passage. In the third passage, the incubation periods for all the L-type BSE isolates converged at about 145 d. These results suggest that the L-type BSE isolates in the primary passage were not fully adapted to the TgBoPrP mice. Furthermore,

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Table 1. *Transmission of L-type BSE isolates in TgBoPrP mice*

	Incubation period (days)			
	JPN	**CAN**	**GER**	**FRA**
First passage	197.7 (3.4)†	172.8 (4.0)*	173.3 (3.3)*	175.7 (5.6)*
	(10/10·)	(12/12)	(12/12)	(10/10)
Second passage	152.0 (1.7)	145.7 (1.8)	143.1 (5.7)	143.1 (3.9)
	(24/24)	(23/23)	(18/18)	(18/18)
Third passage	145.1 (3.6)	143.7 (4.6)	145.3 (8.6)	141.6 (4.7)
	(21/21)	(25/25)	(12/12)	(20/20)

†Mean (standard deviation); ·, Number of affected mice/number of inoculated mice; *$p < 0.05$ for Japanese L-type BSE isolate vs. other L-type BSE isolates in the first passage (Student's t-test).

the different incubation periods in the first passage may be caused by the lower titer of the Japanese L-type BSE prion.

Neuropathological examination of the L-type BSE isolate-affected TgBoPrP were performed using previously described methods.[18] Lesion profile analysis revealed that the degree of brain vacuolation due to the Japanese L-type BSE isolate was similar to that caused by the other L-type BSE isolates (**Fig. 1A**). All the L-type BSE isolates caused severe spongiform changes in the hippocampus, septal nuclei of the paraterminal body and cerebral cortex. We next examined the PrP^{Sc} deposition pattern in the brain using paraffin-embedded tissue (PET) blot, as described previously in reference 18. The distributions of PrP^{Sc} deposits in Japanese L-type BSE isolate-inoculated mice were similar to that of mice inoculated with the other L-type BSE isolates; fine punctate and fine granular PrP^{Sc} were predominantly and uniformly distributed in the pons, cerebellar medulla, midbrain, thalamus and corpus callosum (**Fig. 1B**). Furthermore, similar PrP^{Sc} deposits and distribution patterns were observed in the brain in the first and subsequent passages of all the L-type BSE isolates (data not shown).

We further examined the biochemical properties of PrP^{Sc}, such as the glycoform ratio and molecular mass of proteinase K (PK)-digested PrP^{Sc} (PrPcore). PrP^{Sc} were extracted from the brain of L-type BSE isolate-affected TgBoPrP using previously described methods in reference 18. Western blotting analysis revealed that the glycoform patterns and molecular mass of the PrPcore of the Japanese L-type BSE isolate resembled that of the other L-type BSE isolates. In contrast, clear differences were observed between C-BSE and L-type BSE isolates (**Fig. 2A and B**). Next, we examined the relative PK resistance of PrP^{Sc} from L-type BSE isolate-affected TgBoPrP, as described previously in reference 18. The PrP concentration of the sample was adjusted using the signal intensity of western blot. The PK resistance of PrP^{Sc} from the Japanese L-type BSE was similar to that of the foreign L-type BSE isolates. The PrP^{Sc} of C-BSE-affected TgBoPrP was resistant to digestion with 1,000 μg/ml of PK. In contrast to C-BSE, the PrP^{Sc} signal from the L-type BSE isolates decreased when digested with 500 μg/ml of PK (**Fig. 2C**).

The analyses of L-type BSE cases have been performed using different bovinized PrP-overexpressing transgenic mice, such as TgBoPrP,[18] Tgbov XV[11,21] and Tg540.[22] Thus, it has been impossible to compare the properties of L-type BSE isolates in detail. In this study, therefore, we performed a transmission study of the L-type BSE isolates using identical bovinized PrP-overexpressing transgenic mice to further characterize the Japanese L-type BSE prion. All the L-type BSE isolates transmitted to TgBoPrP, and their incubation periods converged at approximately 145 d following serial passages (**Table 1**). Similar degrees of vacuolation and PrP^{Sc} deposition patterns in the brain were observed among the L-type BSE isolates (**Fig. 1A and B**). Besides the biological characteristics, no differences were observed in the biochemical characteristics of PrP^{Sc} from the L-type BSE isolates (**Fig. 2A–C**). These findings suggest that the examined L-type BSE cases were caused by prions with identical characteristics.

Italian L-type BSE cases are called bovine amyloid spongiform encephalopathy (BASE). We could not compare the characteristics of the Japanese L-type BSE with those of the Italian isolates. In a transmission study using transgenic mice, the French L-type BSE isolate and BASE exhibit similar biological characteristics.[22] Our data indicated that the properties of the Japanese L-type BSE prion are identical to those of the French L-type BSE isolate. It has also been reported that the characteristics of Japanese L-type BSE isolate closely resemble those of BASE in an experimental transmission study in cattle.[19]

The origin of L-type BSE prion is unknown. The present study showed that the Japanese and Canadian L-type BSE prions are identical to those from the European cases. The fact that identical L-type BSE prions exhibit a worldwide distribution is important insight for devising atypical BSE control measures.

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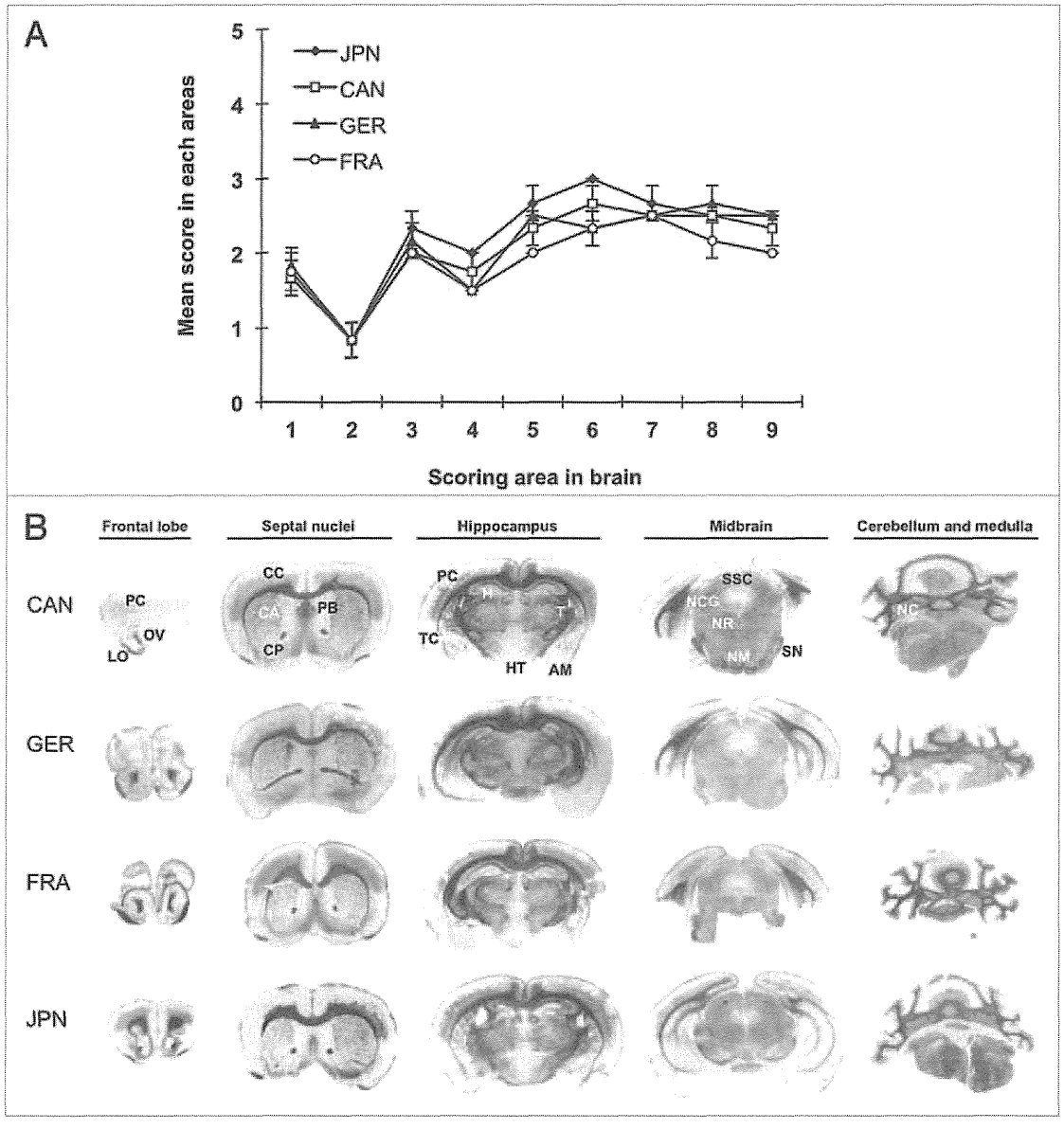

Figure 1. Neuropathological analysis of L-type BSE isolate-affected TgBoPrP. (A) Lesion profile in the first passage. The vacuolation in the following brain regions was scored on a scale of 0–5 (mean values): 1, dorsal medulla; 2, cerebellar cortex; 3, superior cortex; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septal nuclei of the paraterminal body; 8, cerebral cortex at the levels of the hypothalamus and thalamus; and 9, cerebral cortex at the level of the septal nuclei of the paraterminal body. The data are presented as mean ± standard deviation (n = 5). ◆, Japanese L-type BSE (JPN); □, Canadian L-type BSE (CAN); ▲, German L-type BSE (GER); ○, French L-type BSE (FRA). (B) The neuroanatomical distribution of PrP^{Sc} in the brain of TgBoPrP mice infected with Canadian (CAN), German (GER), French (FRA) and Japanese (JPN) L-type BSE isolate by PET-blot analysis. The PET-blot analysis reveals preferential and intense PrP^{Sc} immunolabeling along with periventricular areas, corpus callosum and cerebellar gray matter. Widespread PrP^{Sc} immunolabeling is also detected in the thalamic and brainstem nuclei, while PrP^{Sc} immunostaining in the cerebral and cerebellar cortices and basal ganglia is less conspicuous. Dewaxed membranes were treated with PK (80 μg/mL), followed by denaturation with 3 M guanidine thiocyanate. The monoclonal antibody (mAb) SAF84 was used. Blots corresponding to the brain areas at the level of frontal lob, septal nuclei, hippocampus, midbrain and medulla and cerebellum. FC, frontal cortex; OV, olfactory ventricle; LO, lateral orbital cortex; CC, cingulated cortex; CP, caudate putamen; PB, paraterminal body; CP, caudate putamen; PC, parietal cortex; TC, temporal cortex; H, hippocampus; T, thalamus; HT, hypothalamus; AM, amygdala; SSC, stratum moleculare of the cerebellum; NCG, nucleus corporis geniculati; NR, nucleus rubber; SN, substantia nigra; NM, nucleus mammillaris; NC, deep nuclei of the cerebellum.

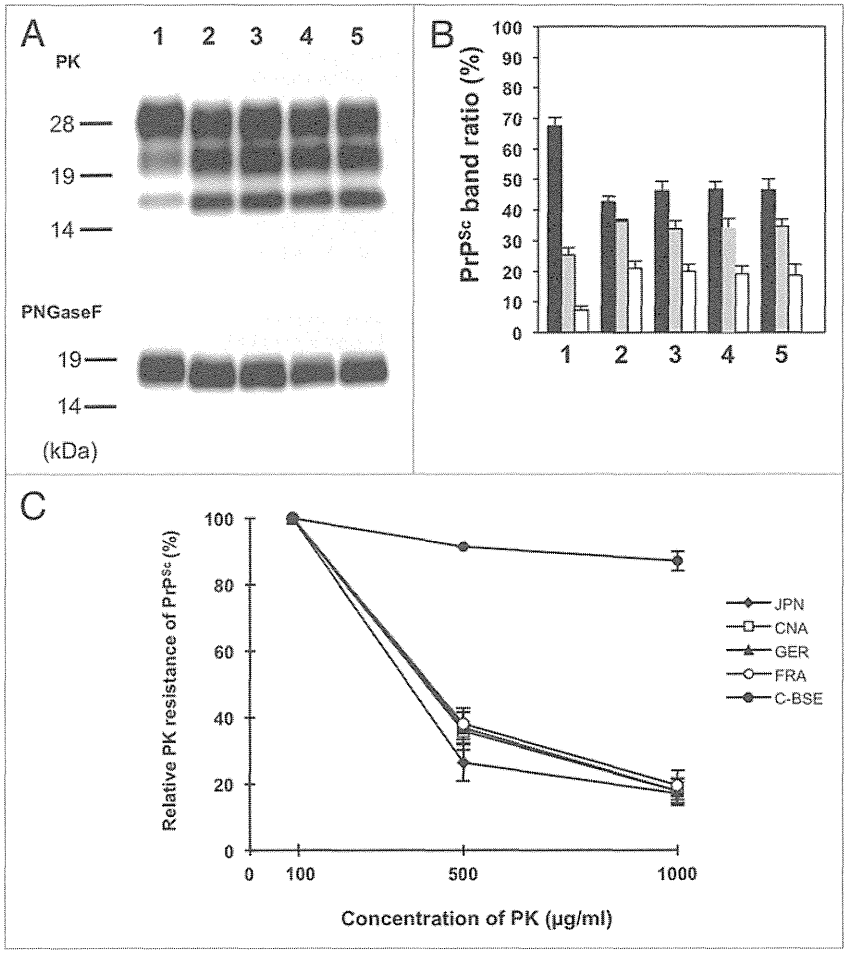

Figure 2. Western blot analysis of proteinase K (PK)-digested prion protein (PrPcore) from the brain of L-type BSE isolate-affected TgBoPrP. (A) Lane 1, Classical-BSE; Lane 2, Japanese L-type BSE; Lane 3, Canadian L-type BSE; Lane 4, French L-type BSE; Lane 5, German L-type BSE. All the samples were digested with 50 μg/ml PK at 37°C for 1 h (upper part), and digested aliquots were treated with N-glycosidase F (PNGaseF), according to the manufacturer's instructions (bottom part). PrPcore was detected with mAb 6H4. Molecular markers are shown on the left (kDa). (B) The relative amounts of the diglycosylated (solid black bar), monoglycosylated (gray bar), and unglycosylated (clear bar) forms in the PrPcore from the brain of L-type BSE isolate-affected TgBoPrP. The lane numbers are as listed in (A). The results are presented as mean ± standard deviation from 5 experiments. (C) Relative PK resistance of PrP^{Sc} from L-type BSE isolate-affected TgBoPrP. The PrP^{Sc} concentration of the sample was adjusted using the western blot signal intensity. The samples were treated with various concentrations of PK (100–1,000 μg/mL). The results are presented as mean ± standard deviation from 3 experiments. PrP^{Sc} was detected with mAb 6H4. ◆, Japanese L-type BSE (JPN); □, Canadian L-type BSE (CAN); ▲, German L-type BSE (GER); ○, French L-type BSE (FRA); ●, Classical-BSE (C-BSE).

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V.　平成 24 年度活動状況

平成24年度活動状況

2012年7月7日

BSE熟議場 in 帯広（アクターの協働による双方向的リスクコミュニケーションのモデル化研究）後援（専門家派遣）

2012年7月29-30日

Asian Pacific Prion Symposium 2012後援

2012年10月18日

平成24年度北海道家畜保健衛生総合検討会（講師派遣）

2013年1月25日

平成24年度第1回研究班会議

2013年3月18日

日本獣医師会　平成24年度　獣医公衆衛生講習会（講師派遣）