Journal of General Virology (2011), **92**, 222–232 **DOI** 10.1099/vir.0.026153-0

Differentiation of ruminant transmissible spongiform encephalopathy isolate types, including bovine spongiform encephalopathy and CH1641 scrapie

J. G. Jacobs,[1] M. Sauer,[2] L. J. M. van Keulen,[1] Y. Tang,[2] A. Bossers[1] and J. P. M. Langeveld[1]

Correspondence
J. P. M. Langeveld
jan.langeveld@wur.nl

[1]Department of Infection Biology, Central Veterinary Institute of Wageningen UR, PO Box 65, 8200 AB Lelystad, The Netherlands

[2]Department of Molecular Pathogenesis and Genetics, Veterinary Laboratories Agency-Weybridge, Woodham Lane, New Haw, Addlestone, Surrey KT15 3NB, UK

With increased awareness of the diversity of transmissible spongiform encephalopathy (TSE) strains in the ruminant population, comes an appreciation of the need for improved methods of differential diagnosis. Exposure to bovine spongiform encephalopathy (BSE) has been associated with the human TSE, variant Creutzfeldt–Jakob disease, emphasizing the necessity in distinguishing low-risk TSE types from BSE. TSE type discrimination in ruminants such as cattle, sheep, goats and deer, requires the application of several prion protein (PrP)-specific antibodies in parallel immunochemical tests on brain homogenates or tissue sections from infected animals. This study uses in a single incubation step, three PrP-specific antibodies and fluorescent Alexa dye-labelled anti-mouse Fabs on a Western blot. The usual amount of brain tissue needed is 0.5 mg. This multiplex application of antibodies directed towards three different PrP epitopes enabled differential diagnosis of all established main features of classical scrapie, BSE and Nor98-like scrapie in sheep and goats, as well as the currently known BSE types C, H and L in cattle. Moreover, due to an antibody-dependent dual PrP-banding pattern, for the first time CH1641 scrapie of sheep can be reliably discriminated from the other TSE isolate types in sheep.

Received 4 August 2010
Accepted 11 October 2010

INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal infectious neurological disorders that can affect humans and animals. Sometimes their pathogenesis is unknown, as in most cases of sporadic Creutzfeldt–Jakob disease (CJD) in men. Often, and especially in animals like sheep, goat, deer and cattle, transmission of TSEs is through oral contact with contaminated food or the environment, or through vertical transmission (Aguzzi, 2006; Prusiner, 1998; Weissmann, 2004). While the TSE sheep scrapie has been known throughout Europe for centuries, bovine spongiform encephalopathy (BSE) was first recognized in 1986, in the UK. The disease and the ensuing epidemic is thought to have stemmed from dissemination of infectious agent in cattle feed containing ruminant-derived protein, which had been inadequately inactivated in destructor plants (Wells *et al.*, 1987; Wilesmith *et al.*, 1988). The consequences were catastrophic since the BSE epidemic not only gave rise to nearly 200 000 clinical cases in cattle in the UK, but had a more global impact on livestock production and trade when it spread to many countries. Furthermore, BSE is understood to be the cause of a new human prion disease known as variant CJD (vCJD), which up until now has been responsible for the death of more than 200 people worldwide (Ironside, 2010).

While scrapie in sheep and goats is not considered to be a human zoonosis, the fear for subclinical BSE in these animals has led to several costly measures, especially in Europe, to protect humans and animals against BSE contamination and to eradicate or better control the disease. Measures include allowing only certain animal products to enter the food chain, testing of all cattle above 30 months of age at death for the presence of BSE, and testing a selection of slaughtered and fallen stock sheep and goat above the age of 18 months (Europeancommunity, 2001, 2005). The success of the measures is evident from the dramatic decline in the incidence of BSE and, in recent years, the substantial reduction of ruminant TSE and human new vCJD cases.

Prion diseases are characterized by the presence and deposition of abnormal forms of the host-encoded cellular glycoprotein prion protein (PrP^{C}), called PrP^{Sc}, after the prion disease archetype scrapie. PrP^{C} occurs relatively universally as a glycosylphosphatidylinositol-anchored

Supplementary tables are available with the online version of this paper.

glycoprotein in the plasma membrane of cells, although its exact function is still unknown (Nicolas *et al.*, 2009). Prion disease diagnosis can be accomplished by testing brain or lymphoid tissues for the presence of PrP^{Sc}, which unlike PrP^{C} is partially resistant to protease digestion. After digestion with serine endoproteases (usually proteinase K, PK), the resistant part of PrP^{Sc} (PrP^{res}) can be observed by Western blotting usually in the 17–30 kDa region as a triple band, representing the di-, mono- and aglycosyl PrP isoforms (Haraguchi *et al.*, 1989; Oesch *et al.*, 1985).

Both BSE and scrapie occur as different types and strains. Strains of TSEs are defined by their phenotypic behaviour after cloning the isolate in rodents (Biacabe *et al.*, 2007; Bruce *et al.*, 2002; Buschmann *et al.*, 2006; Di Bari *et al.*, 2008; Fraser & Dickinson, 1968; Le Dur *et al.*, 2005; Thackray *et al.*, 2008), although the original isolate phenotype frequently is strain indicative. In sheep four TSE types have been recognized based on PrP^{res}-banding patterns: classical scrapie, Nor98 scrapie (a form of atypical scrapie), BSE and CH1641 scrapie (Arsac *et al.*, 2007; Baron *et al.*, 2000; Benestad *et al.*, 2003; EFSA, 2005; Hope *et al.*, 1999; Klingeborn *et al.*, 2006; Stack *et al.*, 2002). In cattle C-type, H-type and L-type BSE have been recognized (Biacabe *et al.*, 2004; Casalone *et al.*, 2004; Jacobs *et al.*, 2007). Due to the remote possibility that BSE may be present but undiagnosed in small ruminants, there is a need for diagnostic methods that clearly differentiate these types, so as to provide, with a high level of confidence, assurance that the presence of BSE-like infections can be ruled out (Europeancommunity, 2005). In principle, this can be carried out by immunohistochemistry or biochemical tests using PrP-specific antibodies. Until now, several sections or blots are needed to evaluate partial removal of epitopes and variations in PrP glycoprofile. In Western blots (WB), BSE in sheep can be discriminated from classical scrapie in sheep (Baron *et al.*, 2000; Hill *et al.*, 1998; Nonno *et al.*, 2003; Stack *et al.*, 2002; Tang *et al.*, 2010; Thuring *et al.*, 2004) based on differences in PrP^{res} length in the region of ovine PrP residues 80–99. CH1641 scrapie is an unusual and rare form of sheep scrapie (Foster & Dickinson, 1988), which has N-terminal PrP^{res} features resembling those of BSE, while its PrP^{res} glycosylation characteristics seem slightly different; furthermore its pathological and transmission properties appeared deviant from BSE (Baron *et al.*, 2000; Hope *et al.*, 1999; Jeffrey *et al.*, 2006a; Somerville *et al.*, 1997; Stack *et al.*, 2002). Differential diagnosis has thus necessitated using long duration bioassays in mice (Europeancommunity, 2001, 2005), or immunohistochemical profiling of PrP epitope distribution in different regions of the brain and lymphoid follicles (Jeffrey *et al.*, 2006b; Lezmi *et al.*, 2006; Thuring *et al.*, 2005). More recently, CH1641 after passage in transgenic mice was found by WB to feature two PrP^{res} populations; interestingly, this could be used for discrimination from both classical scrapie and BSE (Baron & Biacabe, 2007). It is possible that such PrP^{res} features may also be evident in CH1641 of original source sheep.

The aim of this paper was to investigate a multiplex immunoblot approach (triplex-WB), for WB detection with a set of antibodies that bind a single protein (PrP) and are targeted at three different regions A, B and C with the respective epitope amino acid sequences 93–97, 148–153 and 166–172 [ovine PrP sequence coding (Goldmann *et al.*, 1990)] so as to differentially recognize TSE type-specific PrP^{res} fragments (Biacabe *et al.*, 2007). Three murine mAbs, targeting with high affinity amino acid residues in the N-terminal, core and C-terminal domains of ruminant PrP^{res} (Gavier-Widen *et al.*, 2008; Jacobs *et al.*, 2007; Langeveld *et al.*, 2006; Polak *et al.*, 2008), were thus employed to investigate the possibility of establishing a clear-cut discrimination between all current main types of TSEs in sheep and cattle (Fig. 1). Use of various fluorescent anti-mouse Fab–Alexa conjugates enabled simultaneous

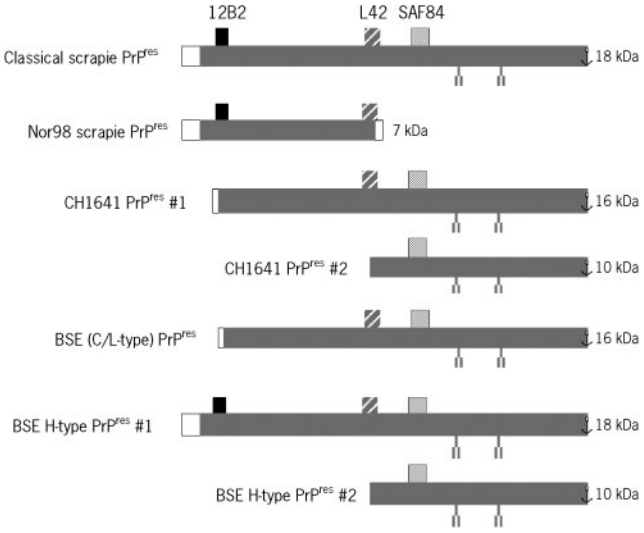

Fig. 1. Schematic diagram of major PrP^{res} bands appearing in WB of various TSE types and the three epitope positions used for this study. PrP^{res} bars approximately reflect the PK-resistant PrP regions observed in classical scrapie, Nor98 scrapie, CH1641 scrapie, C-type BSE, L-type BSE and H-type BSE. The position of ragged ends of PrP^{res} fragments is indicated by open bar segments. The position of the antibody-binding sites if present are indicated for 12B2 (black, IgG1), L42 (hatched, IgG2a) and SAF84 (shaded, IgG2b). These antibodies are from groups A, B and C, respectively, as described previously (Biacabe *et al.*, 2007). The kDa values of the fragments are calculated from the amino acid residue weights for the estimated ovine PrP^{res} sequences in the TSE types. CH1641 scrapie differs from classical scrapie in that the 12B2 epitope is largely absent. PrP^{res} in H-type BSE and also supposedly CH1641 as based on the mouse bioassay data (Baron & Biacabe, 2007) exists of two differently sized triple-band fragments called PrP^{res}#1 and PrP^{res}#2. Position of potentially present glycosyl groups is depicted (fork-like structures), as well as the position of a glycosylphosphatidylinositol group (anchor). Furthermore, a 7 kDa PrP^{res} fragment recognized by 12B2 and L42 as a component in bovine BSE samples is not depicted here (Jacobs *et al.*, 2007).

measurement of binding of the three mAbs to PrP^{res}. The discriminatory power of the triplex-WB was evaluated by application to brain samples from sheep, cattle and goat affected by various scrapie and BSE types.

RESULTS

Fluorescent labels, instrument filters and test sensitivity

Discrimination of scrapie and BSE on a single blot was possible without recourse to signal amplification (e.g. by use of enzyme-labelled secondary antibodies) by use of fluorescently labelled secondary antibody conjugates. This provided a suitable level of sensitivity, stability and emission signal separation (zero cross-talk between different channels) to enable triplex measurements. For detection of the emission signals of Alexa488 or Alexa647, comparison of measurements with the dyes individually or when all three were combined, indicated that there was no significant level of interference (<1 %). For Alexa555, the interfering contribution of Alexa488 in the 580 emission wavelength channel was between 0.5 and 6 % of the signal produced by the Alexa555-antibody conjugate alone. The sensitivity of the method was considered as acceptable for the intended purpose; the lower limit of detection was 2 ng rec-bovinePrP, only a factor of two less sensitive than when enzyme-labelled secondary antibody conjugates were used (data not shown). This sensitivity was mainly accomplished thanks to an effective background reduction using only PVDF membranes around the gels during electrotransfer.

Linearity and assay variation

Triplex-WB measurement of PrP^{res} in brain homogenate from TSE-affected animals diluted threefold in homogenate from negative control animals exhibited highly linear dose–response correlations (r^2=0.9941–0.9997, Fig. 2). This indicated that data can be reliably compared between samples in working ranges of 0.01–0.5 mg tissue equivalents per lane. As a further indicator for assay performance, imprecision within and between tests was calculated by considering the 12B2/L42 ratios. Within-assay coefficient of variation (CV%), defined as testing the same sample threefold in three different SDS-PAGE runs with subsequent blotting and immunotesting was between 11 and 25 % (Table 1). The between-assay CV% for three measurements of the same sample was between 25 and 40 % (Table 1). Between-assay imprecision (CV%) for the glycoprofile determinations were relatively small for the di- and monoglycosyl fractions: ⩽10 % for L42 and ⩽29 % for 12B2 measurements (Table 2). Taking these data together, the triplex-WB appeared reproducible and yielded consistent 12B2/L42 ratios for determination irrespective of sample source (ovine, bovine, scrapie, BSE or CH1641). Similarly, glycoprofiles produced using either L42 or 12B2 were very similar to each other (see Table 2).

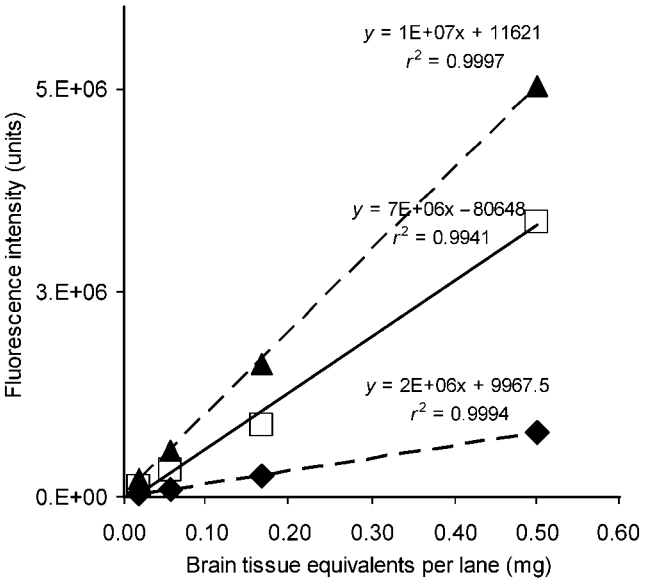

Fig. 2. Dose–response curves for triplex-WB representative for TSE samples. In this example, a moderately scrapie-positive sheep brain homogenate was diluted over a threefold range with negative control brain homogenate. The mixtures were digested with PK and, after purification by 1-propanol precipitation, were analysed on SDS-PAGE and blotting using the antibody triplex as described: 12B2 (▲), SAF84 (□) and L42 (◆). The lines of best fit and co-efficients of determination (r^2) were determined by linear least square regression analysis.

Triplex-WB patterns of different TSE isolates

The triplex-WB was applied to sample sets representing various TSE types from both sheep (classical scrapie, Nor98 scrapie, CH1641 scrapie and experimental BSE) and cattle (C-type, H-type and L-type). Triplex-WB assays of sheep classical scrapie, BSE and CH1641 scrapie demonstrated the well established triple-banding pattern in the molecular mass 17–30 kDa range contrasting with a major 7.6 kDa distinctive discriminative 12B2- and L42-positive bands in Nor98 scrapie, which frequently also exhibited a 14.4 kDa band (Fig. 3a; Supplementary Table S2, available in JGV Online). Binding with mAbs 12B2, L42 and SAF84 was relatively strong for classical scrapie PrP^{res} (Fig. 3a, lane 2), and although for mAbs L42 and SAF84 similarly strong binding was evident for ovine experimental BSE and CH1641 scrapie PrP^{res}, that for 12B2 was relatively weak in these two types (Fig. 3a, lanes 3 and 4). Interestingly, CH1641 displayed a clearly different banding pattern from L42 and SAF84 with L42 exhibiting a diglycosylated PrP^{res} fraction that is the predominant band, and with SAF84 having roughly equal intensities between di- and monoglycosylated PrP^{res} (Fig. 3a, compare all lanes 4). In cattle, L-type and C-type BSE exhibited minimal or absent staining with 12B2 compared with that of L42 and SAF84 (Fig. 3b, lanes 1 and 2), while 12B2 staining in H-type was relatively intense (Fig. 3b, all lanes 3). Similar to what was

Table 1. Within- and between-test imprecision of 12B2/L42-binding ratios that reflect the extent of N-terminal cleavage of PrP^{res}

12B2/L42 ratios represent the proportion of antibody binding (fluorescence units) for antibodies 12B2 and L42. These values are relative, and are not absolute indicators of the number of epitopes of 12B2 to L42 in PrP^{res}. In the column heading are indicated the type of isolate, its signal strength in previous tests using chemiluminescence detection (Jacobs *et al.*, 2007), and animals identity number which can be used for sample identification in Supplementary Table S1 (available in JGV Online). Ratio values are means ± SD, with imprecision (CV%) values in parentheses. Antibodies used: L42 (0.1 μg ml^{-1}), detection label Alexa647; 12B2 (0.1 μg ml^{-1}), detection label Alexa488.

Imprecision	Ov scrapie strong 2005-18	Ov scrapie moderate 2006-32	Bo BSE strong NL-36	Ov BSE strong 6019325	Ov CH1641 moderate J2935	Ov CH1641 moderate J3011
Within-test	6.2 ± 0.7* (11)	8.2 ± 1.9* (23)	0.4 ± 0.1† (25)	0.4 ± 0.1* (25)	1.8 ± 0.4* (22)	1.4 ± 0.2‡ (14)
Between-test	8.8 ± 2.2§ (25)	9.8 ± 3.2§ (33)	0.5 ± 0.2§ (40)	0.5 ± 0.2§ (40)	3.5 ± 1.2§ (34)	2.4 ± 0.9§ (38)

*†, ‡Within-test imprecision estimation was performed nine times on the same sample in NuPAGE loading buffer, but in the two cases † and ‡ the number is 5 and 8, respectively; the missing values were due to too low 12B2 values (below the cut-off). In these latter two cases the results were anyway indicative for a BSE-like state.

§Between-test imprecision estimation was obtained by three successive experiments each with a new digestion with PK, SDS-PAGE run and triplex-WB.

noticed for CH1641 with respect to differentiating banding patterns between L42 and SAF84, also H-type staining yielded a difference in glycoprofile with a predominant diglycosylated band with L42 and equal intensities between di- and monoglycosylated PrP^{res} with SAF84 (Fig. 3b, compare all lanes 3). The different glycoprofiles obtained between mAbs L42 and SAF84 within single samples of CH1641 scrapie and H-type BSE is further referred to as 'dual glycotype'.

Discriminatory analysis of isolate types from sheep, cattle and goat

Evaluation of differences between antibody-binding profiles for several subjects of each isolate type demonstrated reproducible outcomes and reliable differential diagnosis based on several parameters: (i) 12B2/L42 ratio as N-terminal marker for PK sensitivity of PrP^{res} (Fig. 4), (ii) relative dominance of diglycosyl moieties using L42 (Fig. 5a), as well as (iii) SAF84/L42 ratio at 24 kDa position as a marker for the potential presence of different antibody-dependent glycoprofiles of PrP^{res} or shortly dual glycotype (Fig. 5b).

For ovine isolates, the discriminating power of the 12B2/L42 ratio was on average 12-fold between ovine scrapie and BSE, and somewhat smaller (6-fold) between ovine scrapie and CH1641 (Fig. 4). The discriminating power between CH1641 and ovine classical scrapie and BSE was further strengthened by a dual glycoprofile aspect in only CH1641 when comparing the L42 and SAF84 staining (Fig. 5b). The two goat-scrapie samples examined gave similar binding profiles to those observed for classical sheep scrapie.

For bovine isolates, the discriminative power of the 12B2/L42 ratio was on average 14-fold between H-type BSE, and C-type and L-type BSE (Fig. 4). The PrP^{res} glycoprofile of L-type BSE when tested with L42 was strikingly different from that of H-type and C-type BSE in that the proportions of the di- and monoglycosylated PrP^{res} fractions were nearly equal (Fig. 5a). The H-type cases, however, clearly exhibited antibody-dependent glycoprofiles of PrP^{res} by SAF84/L42 ratios which demonstrated that a substantial fraction of signal was located at the monoglycosylated PrP^{res} position migrating at 24 kDa (Figs 3 and 5b).

Table 2. Between-test imprecision of glycoprofile estimations

Glycoprofiles were determined in three different experiments. The samples tested are from the same experiment as the between-test experiment of Table 1. Values for di-, mono- and aglycosylated PrP^{res} are expressed as percentages of the total PrP^{res} signal, and as means ± SD, with imprecision (CV%) values in parentheses.

	PrP^{res}- moiety	Ov scrapie 'strong'	Ov scrapie 'moderate'	Bo BSE 'strong'	Ov BSE 'strong'	Ov CH1641 'moderate'	Ov CH1641 'moderate'
L42	Di-	56 ± 2 (4)	53 ± 2 (4)	69 ± 3 (4)	67 ± 1 (1)	60 ± 2 (3)	62 ± 3 (5)
	Mono-	28 ± 2 (7)	30 ± 1 (3)	20 ± 2 (10)	21 ± 1 (5)	30 ± 1 (3)	30 ± 1 (3)
	A-	15 ± 1 (7)	17 ± 1 (6)	11 ± 1 (9)	12 ± 1 (8)	10 ± 1 (10)	8 ± 2 (25)
12B2	Di-	56 ± 2 (4)	53 ± 2 (4)	68 ± 7 (10)	65 ± 7 (11)	57 ± 4 (7)	62 ± 2 (3)
	Mono-	28 ± 2 (7)	31 ± 2 (6)	24 ± 7 (29)	22 ± 4 (18)	28 ± 1 (4)	27 ± 1 (4)
	A-	17 ± 2 (12)	16 ± 1 (6)	8 ± 1 (13)	12 ± 3 (25)	15 ± 3 (20)	11 ± 2 (18)

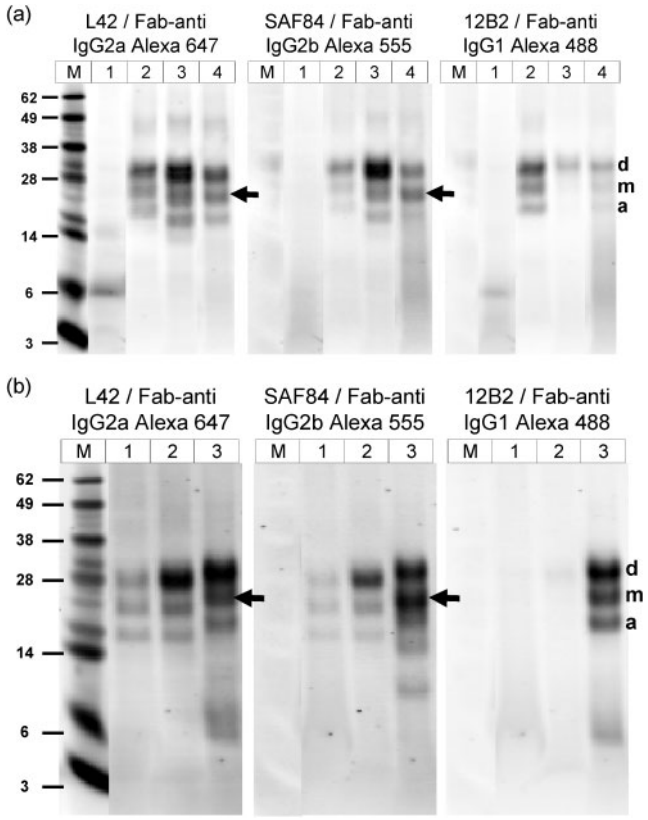

Fig. 3. PrP^{res} from ovine (a) and bovine (b) TSE isolate types visualized by triplex-WB. (a) Lanes 1, Nor98 scrapie; lanes 2, classical scrapie; lanes 3, ovine BSE; lanes 4, CH1641 scrapie. (b) Lanes 1, L-type BSE; lanes 2, C-type BSE; lanes 3, H-type BSE. Arrows indicate the position of the 24 kDa migration position. Symbols d, m and a indicate the migration positions of di-, mono- and aglycosylated forms of PrP^{res}. Each of the three antibody combinations used is indicated above the triplex-WB generated lane-sets.

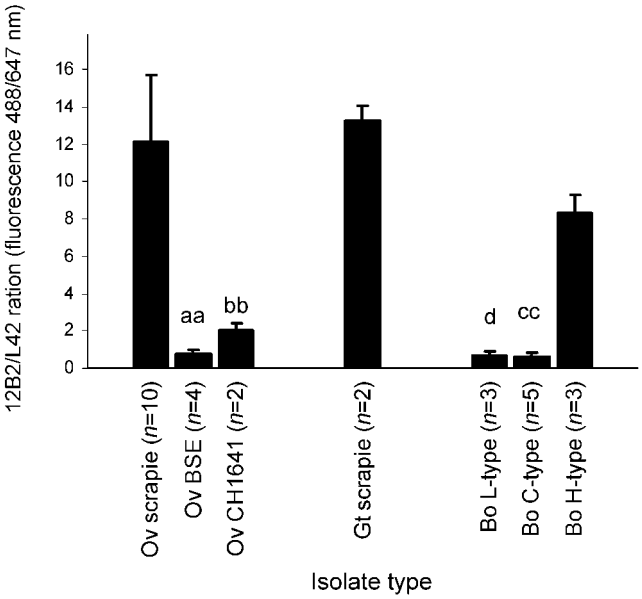

Fig. 4. Comparing the presence of N terminus of PrP^{res} in different isolate types from sheep (Ov), goat (Gt) and cattle (Bo) by triplex-WB analysis. A high 12B2/L42 ratio reflects a relative strong resistance of the PrP^{Sc} N terminus to proteolysis. The isolate types used are indicated. The origin of these isolates are detailed in Supplementary Table S1. Values represent mean ± SD (n=no. cases tested). Differences between ratios for ovine- and goat-scrapie isolate types compared with experimental ovine BSE (aa, $P<0.001$) and CH1641 (bb, $P<0.001$, only two samples) were statistically significant. Similarly, the ratio for bovine BSE H-type was statistically different from those for C-type (cc, $P<0.001$) and L-type (d, $P<0.01$).

DISCUSSION

The triplex immunostaining method – triplex-WB – described here is novel in its application for analysing different epitopes of a single protein in a single blot. Importantly, this study contributes substantially to enhancing the capability for rapid differential diagnosis of TSE types in field isolates from sheep and cattle.

The study showed that data generated from several antibodies with distant PrP-epitope locations can be interpreted in a simple, sensitive and reliable manner to enable differential diagnosis of TSE types in sheep and cattle. This was possible for several practical reasons. Firstly, dose–response studies demonstrated a linear relationship between tissue concentration and binding of the triplex target antibodies 12B2, L42 and SAF84, indicating the possibility of maintaining differential diagnostic capability in a manner relatively independent of the content of PrP^{Sc} present in the sample. Secondly, the triplex-WB procedure using non-enhanced fluorescently labelled reagents exhibited little loss of sensitivity (by a factor of two) compared with enzymic enhancement procedures. This was accomplished thanks to the stability of the labels, and to the low backgrounds on the blot membranes. Also, there is no substrate diffusion which with enzymically produced end products can hamper quantification. Nevertheless, the triplex-WB system requires care for preventing background problems by assuring absence of precipitates in the reagents, as well as by omitting protein-blocking reagents. Thirdly, the principle of simultaneous measurement of binding of three different antibodies on the same blot and the same protein reduces the requirement for measuring several membranes. As a consequence errors associated with differences in analyte migration between the gels and other procedural variations can be eliminated or minimized. To be able to perform the triplex-WB technique it is obvious that for measuring three fluorescent labels a high-tech imager is required with three different lasers for excitation, as well as appropriate optical technology.

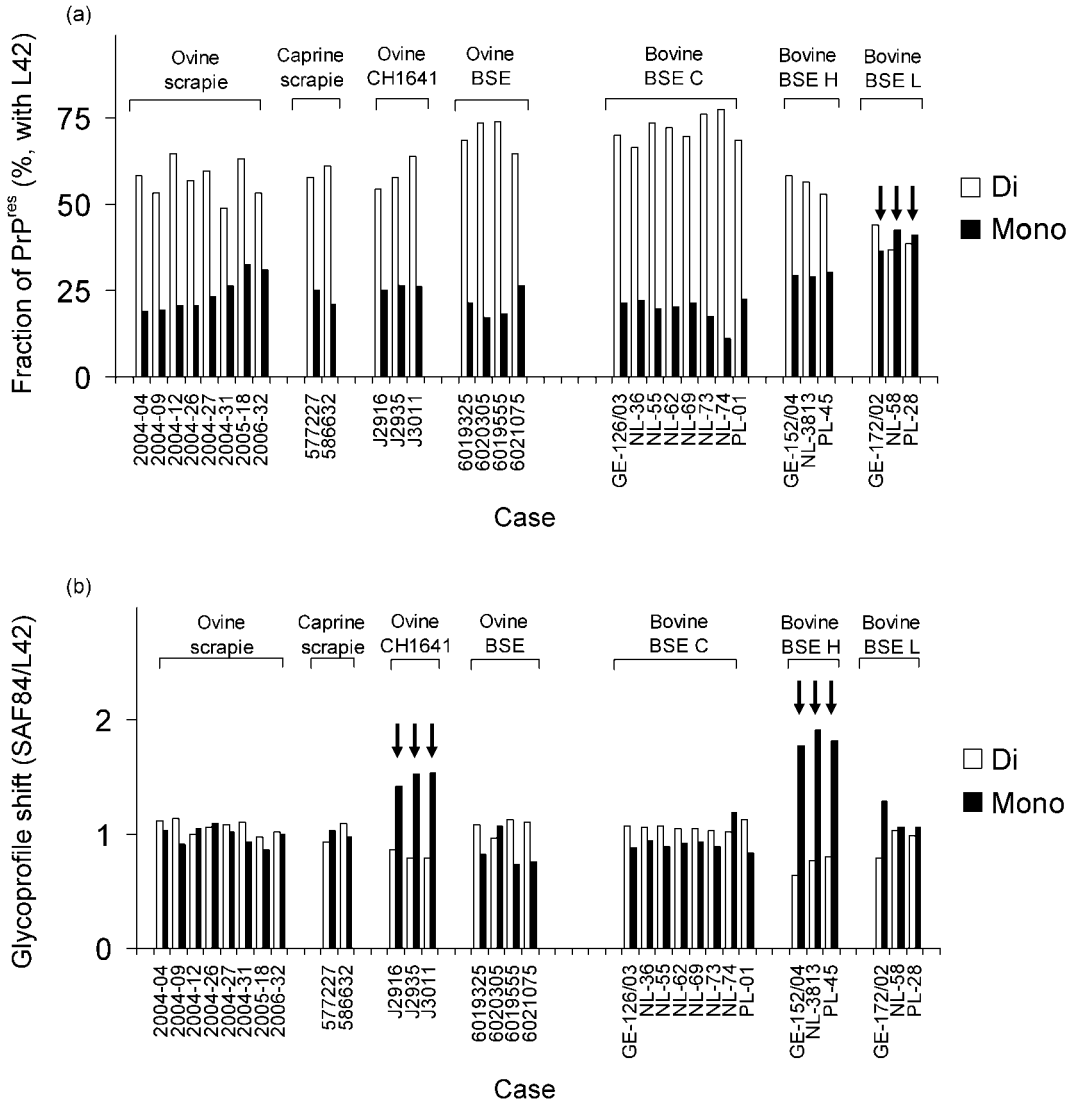

Fig. 5. PrP^{res} glycoprofile of sheep and cattle TSE isolates characterized using antibodies L42 and SAF84 in triplex-WB. (a) Relative abundance of di- and monoglycosylated PrP^{res} for individual ovine and bovine TSE cases, detected by mAb L42. Values are percentages per PrP^{res} band. Only in case of L-type BSE di- and monoglycosyl moieties occur in near equal proportions (arrows). In contrast, the diglycosyl moiety is the predominant PrP^{res} form in classical sheep and CH1641 scrapie, and in cattle C-type and H-type BSE. (b) The ratio of SAF84/L42 binding to mono- and diglycosylated PrP^{res} in TSE isolates. Only for CH1641 scrapie and H-type BSE there was evidence of a substantial difference between binding associated with the monoglycosylated PrP^{res} form (dual glycotype antibody-binding behaviour, arrows), thus concerning the band at the 24 kDa position (arrowed in Fig. 3).

TSE type features in small ruminants

In the current study, the molecular differentiation in sheep by WB between classical scrapie, Nor98 scrapie and BSE in small ruminants was as effective as in the current methods for discrimination. Nor98 scrapie PrP^{res} is characterized by the presence of a major 7–8 kDa band that is reactive with group A antibody 12B2 (comparable to the species-dependent mAb P4 used elsewhere), and group B antibody L42 (Benestad *et al.*, 2003; Biacabe *et al.*, 2007; Gretzschel *et al.*, 2006; Klingeborn *et al.*, 2006; Polak *et al.*, 2010). Classical scrapie can be recognized by the equally strong reactivity between group A and B antibodies 12B2 and L42 (or between P4 and 6H4) (Langeveld *et al.*, 2006; Stack *et al.*, 2002; Thuring *et al.*, 2004). However, until now it has not proved readily possible to distinguish PrP^{res} from CH1641 scrapie and ovine BSE with respect to virtual absence of the P4-binding epitope, one of the two established hallmarks for BSE cases (Stack *et al.*, 2002). Discrimination between CH1641 scrapie and BSE was

further obscured by the second hallmark for BSE that is a relative predominant diglycosyl fraction (>60 %), while this fraction was slightly lower in CH1641 PrP^{res} using group B antibodies (Baron *et al.*, 1999, 2000; Gretzschel *et al.*, 2005; Lezmi *et al.*, 2004; Stack *et al.*, 2002). Recently, a novel molecular aspect in CH1641 scrapie became obvious after transgenic TgOvPrP4 mice, which express ovine wild-type PrP, had been inoculated with brain material from either CH1641 experimentally infected sheep or some natural unusual ovine scrapie cases (Baron & Biacabe, 2007). It was concluded that this type of isolate carried two populations of PrP^{res}: PrP^{res}#1 and PrP^{res}#2. Since PrP^{res}#1 and PrP^{res}#2 are both recognized by group C antibodies like SAF84, while group B antibodies like L42 only bind to PrP^{res}#1, SAF84 will yield a different glycoprofile from that produced with L42, a phenomenon that has been described for H-type BSE (see Fig. 1 for structural explanation). In keeping with these observations, we also found in PrP^{res} material from CH1641-infected sheep indications for the existence of a dual population of PrP^{res} types. Significant for discrimination, binding profiles for BSE and classical scrapie were similar whether SAF84 or L42 was used, whereas for CH1641 glycoprofiles were characteristically different for the two mAbs. Thus, reliable discrimination appeared possible now between classical scrapie, CH1641 scrapie and BSE in sheep. The finding that experimental CH1641 scrapie in sheep now can be faithfully differentiated from experimental BSE in sheep using this dual glycotype aspect as observed with the mAbs L42 and SAF84 is new and important in decision making when small ruminant TSE surveillance encounters field cases with BSE-like appearance (Eloit *et al.*, 2005; Jeffrey *et al.*, 2006b; Lezmi *et al.*, 2004).

BSE type features in cattle

In cattle samples, single blot triplex-WB yielded consistently the antibody-binding patterns for C-type, L-type and H-type BSE isolates as expected when tested in three separate blots (Jacobs *et al.*, 2007). C-type and L-type isolates showed very limited presence of the 12B2 epitope compared with the L42 core epitope. Furthermore, L-type samples showed a relatively high monoglycosylated fraction of PrP^{res} (signal intensity equal to the diglycosyl fraction) with both antibodies L42 (group B) and SAF84 (group C) (Fig. 5). H-type BSE exhibited relatively elevated levels of the 12B2 binding, indicative for increased resistance to N-terminal cleavage by PK compared with C-type BSE, and in this respect comparable with that found in classical scrapie (Fig. 4). However, H-type BSE, like CH1641 scrapie, was notable by a dual glycotype aspect with antibodies L42 and SAF84 (Fig. 5b).

Discriminators for ruminant TSE isolate typing by PrP^{res} analysis

The extent of which TSE types can be most simply differentiated by triplex-WB is readily illustrated for sheep and cattle by the spatial separation made evident by comparing the relative amounts of N-terminal epitope to core epitope (12B2/L42 ratio) with the binding properties as represented by the SAF84/L42 ratio at the 24 kDa position (Fig. 6a). In sheep, CH1641 can be easily distinguished from BSE by the difference between SAF84/L42 ratio values. Nor98 scrapie is characterized by a main PrP^{res} band at 7–8 kDa (Fig. 3). In cattle, the values for both 12B2/L42 and SAF84/L42 ratios are substantially elevated in H-type BSE compared with C-type and L-type BSE (Fig. 6b). L-type BSE can be discriminated from C-type by equal di- and monoglycosyl moiety fractions (Fig. 5a). It can be envisaged that the technique will be useful in the typing of the human sCJD types as well as the vCJD samples e.g. sCJD type I, will be characterized by a high 12B2 binding (like scrapie and H-type BSE), while sCJD type II differs from vCJD by a relatively low diglycosyl moiety (like L-type BSE) (Notari *et al.*, 2007).

Indeed, the novel triplex-WB procedure proved capable of distinguishing all established forms of TSE isolates in livestock: classical scrapie, Nor98 scrapie, BSE and CH1641 scrapie in sheep and C-type, H-type and L-type BSE in cattle. The assay principle may thus prove invaluable in reducing ambiguity in disease classification and thus assist in reducing the need to perform animal bioassays for strain typing to exclude the potential presence or absence of BSE-like infections. The technique will also be attractive for application in future PrP^{Sc} and PrP^{res} typing efforts for tissue section analyses and *in vitro* assays as well.

METHODS

Chemicals and reagents. Chemicals were purchased from Merck unless otherwise specified, and were at least analytical reagent grade. Reagents for SDS-PAGE and the Molecular Probes Zenon kits for mouse IgG class-specific detection, containing isotype-specific goat anti-mouse-Fc Fab-Alexa Fluor conjugate (Zenon Alexa Fluor 647 anti-Mouse IgG2a, catalogue no. Z25108; Zenon Alexa Fluor 555 anti-Mouse IgG2b, catalogue no. Z25205 and Zenon Alexa Fluor 488 anti-Mouse IgG1, catalogue no. Z25002) were purchased from Invitrogen. Purified recombinant bovine PrP was obtained from Prionics AG, Schlieren, Switzerland.

Antibodies and their epitope specificities. Murine anti-PrP mAbs 12B2 IgG1, L42 IgG2a (L42 appeared to be an IgG2a class antibody, and was erroniously reported as an IgG1 class in Harmeyer *et al.*, 1998) and SAF84 IgG2b were chosen for simultaneous use in triplex-WB (Feraudet *et al.*, 2005; Harmeyer *et al.*, 1998; Langeveld *et al.*, 2006), since these met the potential requirements for TSE type discrimination, exhibited high affinity to ruminant PrP and represented different IgG classes (Fig. 1). The epitope specificities of these mAbs were mapped by Pepscan analyses with solid phase bound 15-mer PrP-based peptides (Langeveld *et al.*, 2006; Slootstra *et al.*, 1996): 12B2, L42 and SAF84 are specific for epitopes in PrP 93WGQGG97, 148YEDRYY153 and 166YRPVDQY172, respectively [ovine PrP sequence numbering (Goldmann *et al.*, 1990)].

Tissues, homogenates, digestion and partial purification. Sheep, goat and cattle brainstem tissues were obtained either from experimental infections or from slaughterhouse or fallen stock material as part of routine screening programmes for TSE; the

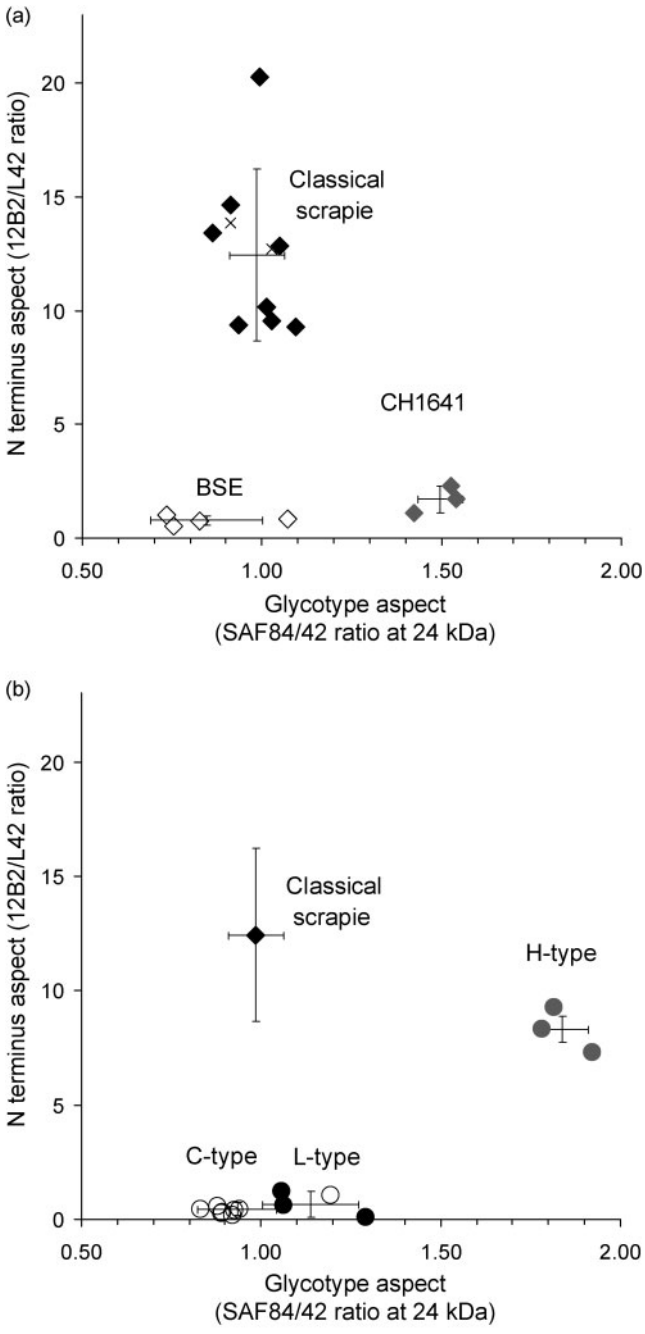

Fig. 6. Diagram to illustrate two PrP^{res} aspects that can provide discrimination between single TSE isolate types in sheep or cattle. The 12B2/L42 ratio on the vertical axis shows the aspect of N-terminal processing in the 12B2 epitope relative to the PrP^{res} core (L42 epitope); the SAF84/L42 ratio on the horizontal axis demonstrates the extent of dual glycotype where SAF84 can bind to all C-terminal fragments of PrP^{res} and L42 to all PrP^{res} fragments except the supposed C-terminal PrP^{res}#2 populations of CH1641 scrapie and H-type BSE (see also Fig. 1). For each dataset of TSE type isolates, also the mean ± SD is shown. L-type BSE can be discriminated from C-type BSE by its high monoglycosyl PrP^{res} content relative to that of the diglycosyl fraction (see Fig. 5a). (a) Data points are from classical scrapie (solid diamonds, $n=8$), experimental BSE (open diamonds, $n=4$) and CH1641-affected sheep (grey diamonds, $n=3$) and two goat with classical scrapie (crossed symbols). (b) Data points are from cattle affected with C-type (open circles, $n=8$), L-type (solid circles, $n=3$) and H-type BSE (grey circles, $n=3$). Ovine classical scrapie from (a) is included as a reference point to facilitate comparisons between panels (a) and (b).

routine preliminary testing of samples was performed using a commercially available WB assay procedure (Prionics-Check Western blot; Schaller *et al.*, 1999). Data on individual samples can be found in a separate table, including German and Polish BSE cases (Supplementary Table S1). The TSE status was further confirmed by immunohistochemistry. Tissue from CH1641 scrapie was prepared through intracerebral challenge in three Cheviot sheep (provided by Foster & Hunter, Edinburgh). First passage and second passage ovine BSE material was prepared by combined intercerebral, intravenous and intraperitoneal inoculation of ARQ/ARQ sheep, starting with bovine BSE brain material TSE-free collected from a Dutch case (NL-62) with clinical signs. Two confirmed positive-scrapie goats from a Dutch recreational city farm were also used, one of which in clinical condition. One H-type BSE sample was generated by intracerebral inoculation of obex material from a Dutch H-type case NL-22 (Jacobs *et al.*, 2007). Confirmed TSE-negative brainstem tissues from a cow and a sheep served as negative controls. TSE-positive tissues were stored at −70 °C and negative tissues at −20 °C. Ten per cent homogenates were prepared in lysis buffer and digested with PK at 37 °C as described previously (Jacobs *et al.*, 2007). Partial PrP^{res} purification was achieved by precipitation in 1-propanol (34871; Sigma-Aldrich) centrifugation at 16 000 ***g*** for 5 min in a Biofuge Pico centrifuge (Heraeus) at room temperature. After discarding the supernatant, pellets contained 10 mg tissue equivalents (TE).

SDS-PAGE and WB. PrP^{res} pellets were resuspended and dissolved in 100 µl NuPAGE loading buffer 1×, including reducing agent just before use [141 mM Tris base (93362; Sigma), 106 mM Tris/HCl (93287; Sigma), 73 mM lithium dodecyl sulfate (62554; Fluka), 1.09 M glycerol (49767; Fluka), 0.51 mM EDTA, without front marker dyes, with NuPAGE Sample Reducing Agent 1× (NP0009; Invitrogen)]. The sample was denatured by heating for 10 min at 96 °C. After cooling down to room temperature, the sample was centrifuged at 16 000 ***g*** for 5 min in a Biofuge Pico microcentrifuge at room temperature. Sample supernatants (5 µl, 500 µg TE per lane) were then subjected to SDS-PAGE by using 17-well NuPAGE 12 % Bistris 1.0 mm precast gels. As molecular mass reference, a 5 µl aliquot from a 50 times dilution of SeeBlue Pre-Stained Standard (LC5625; Invitrogen) was applied to a separate gel lane. Electrophoresis was performed with 2-morpholinoethanesulfonic acid SDS running buffer with antioxidant. Electrotransfer onto minimally fluorescent PVDF membrane with 0.45 µm pore size (Immobilon-FL; Millipore) was performed in a tank-blotter (Trans-Blot Electrophoretic Transfer Cell; Bio-Rad) for 60 min at 150 V with temperature control at 8 °C and pre-cooled transfer buffer pH 8.3 (25 mM Tris, 192 mM glycine, 10 % methanol). A pre-wetted PVDF transfer sheet and the gel was sandwiched between two other sheets of pre-wetted PVDF membrane. After transfer, the blot was washed three times for 5 min in washing buffer [25 mM Tris base, 0.137 M NaCl, 0.003 M KCl, adjusted to pH 7.4 with HCl, 0.5 % (v/v) Tween-20].

Triplex antibody incubation and detection on WB (triplex-WB). The product information protocol from the Zenon Mouse IgG Labelling kits was followed, and a standard 3 : 1 molar ratio Fab : mAb was used. Incubations were performed in the dark at room

temperature. The Zenon Complex Formation protocol was performed in separate tubes for each antibody. Antibodies 12B2, L42 and SAF84 were used at respective concentrations of 0.1, 0.1 and 0.5 μg IgG ml^{-1}. For immunocomplex formation, the appropriate amount of individual purified mAbs and proportion of corresponding Zenon Alexa Fluor-labelled anti-mouse isotype was added to 10 μl washing buffer and mixed. Following a 5 min incubation, an equal amount of Zenon-blocking reagent was added to each, mixed and incubated for 5 min. The three blocked Zenon complexes were then diluted in washing buffer, mixed with each other in the correct ratio, added to the blot and then incubated for 1 h on a rocking platform. Blots were then washed four times for 5 min with washing buffer and left to dry in a free hanging condition in the dark overnight. Dried blots were inserted into development folders (T2258; Applied Biosystems) and visualized by fluorescence scanning (pixel size 200 μm) on a Typhoon Trio variable-mode imager (Amersham Biosciences) set at focal plane platen. Red, green and blue lasers were used for excitation (633, 532 and 488 nm) with corresponding emission filters (670BP30, 580BP30 and 520BP40). For each channel the photomultiplier tube was set at 400 V and normal sensitivity.

Quantification, calculation and statistical analysis. The data files with light emission values from the Typhoon scanner were analysed using ImageQuant software (Molecular Dynamics/Amersham Biosciences) and images were quantified by positioning either rectangular areas around (for mAb ratio calculation) or lines over (for glycoprofile calculation) triple bands in each lane. The light emission values measured in the rectangles were further processed for background correction of each lane using the local median correction option; data were exported to Excel (Microsoft). A mean general background value was established for each blot by placing also four rectangles (blanks) at blot sites with minimal fluorescence. The light emission noise was considered as above background when mean values obtained in the four blanks were >2.5 times general background. For glycoprofile calculation at the line overlays, light emission values measured for each of the three bands of a triple PrP^{res}-band pattern were expressed as a percentage of total light emission of the three bands. Assay imprecision (CV%) for a set of data was calculated as coefficient of variation, which is expressed as [SD/mean × 100 %].

Proprietary software was used for statistical analyses (Instat Biostatistics from Graph-Pad Software). One-way analysis of variance was used to establish whether variations between groups of data (12B2/L42 ratio values) were greater than expected. In this case, differences between pairs of groups were considered statistically significant if probability for equality was <0.05 in multiple-comparisons tests (Student–Newman–Keuls test).

ACKNOWLEDGEMENTS

Financial support for this work was provided by the Department for Environment, Food and Rural Affairs (Defra, UK; project SE1700/CSA 7335). Part of this work was also possible thanks to the EU funded STREP project GoatBSE, FOOD-CT-2006-36353. J. P. M. L. is partly supported by the Dutch Ministry of Agriculture, Nature and Food Quality. The authors appreciate informative discussions with Dr Jacques Grassi and support in the provision of Sha31 antibody. We thank Dr Marielle Melchior for helpful comments when preparing the manuscript, Ing. Jo Erkens for assistance in homogenate preparations and Yvette Bisselink for preliminary development work to determine assay sensitivity. The ovine CH1641 samples were kindly provided by Jim Foster and Nora Hunter, The Roslin Institute, University of Edinburgh, German and Polish BSE brain material were from a previous study (Jacobs *et al.*, 2007).

REFERENCES

Aguzzi, A. (2006). Prion diseases of humans and farm animals: epidemiology, genetics, and pathogenesis. *J Neurochem* **97**, 1726–1739.

Arsac, J. N., Andreoletti, O., Bilheude, J. M., Lacroux, C., Benestad, S. L. & Baron, T. (2007). Similar biochemical signatures and prion protein genotypes in atypical scrapie and Nor98 cases, France and Norway. *Emerg Infect Dis* **13**, 58–65.

Baron, T. & Biacabe, A. G. (2007). Molecular behaviors of "CH1641-like" sheep scrapie isolates in ovine transgenic mice (TgOvPrP4). *J Virol* **81**, 7230–7237.

Baron, T. G., Madec, J. Y. & Calavas, D. (1999). Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. *J Clin Microbiol* **37**, 3701–3704.

Baron, T. G., Madec, J. Y., Calavas, D., Richard, Y. & Barillet, F. (2000). Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. *Neurosci Lett* **284**, 175–178.

Benestad, S. L., Sarradin, P., Thu, B., Schonheit, J., Tranulis, M. A. & Bratberg, B. (2003). Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Vet Rec* **153**, 202–208.

Biacabe, A. G., Laplanche, J. L., Ryder, S. & Baron, T. (2004). Distinct molecular phenotypes in bovine prion diseases. *EMBO Rep* **5**, 110–115.

Biacabe, A. G., Jacobs, J. G., Bencsik, A., Langeveld, J. P. & Baron, T. G. (2007). H-type bovine spongiform encephalopathy: complex molecular features and similarities with human prion diseases. *Prion* **1**, 61–68.

Bruce, M. E., Boyle, A., Cousens, S., McConnell, I., Foster, J., Goldmann, W. & Fraser, H. (2002). Strain characterization of natural sheep scrapie and comparison with BSE. *J Gen Virol* **83**, 695–704.

Buschmann, A., Gretzschel, A., Biacabe, A. G., Schiebel, K., Corona, C., Hoffmann, C., Eiden, M., Baron, T., Casalone, C. & other authors (2006). Atypical BSE in Germany – proof of transmissibility and biochemical characterization. *Vet Microbiol* **117**, 103–116.

Casalone, C., Zanusso, G., Acutis, P., Ferrari, S., Capucci, L., Tagliavini, F., Monaco, S. & Caramelli, M. (2004). Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt–Jakob disease. *Proc Natl Acad Sci U S A* **101**, 3065–3070.

Di Bari, M. A., Chianini, F., Vaccari, G., Esposito, E., Conte, M., Eaton, S. L., Hamilton, S., Finlayson, J., Steele, P. J. & other authors (2008). The bank vole (*Myodes glareolus*) as a sensitive bioassay for sheep scrapie. *J Gen Virol* **89**, 2975–2985.

EFSA (2005). Opinion on classification of atypical transmissible spongiform encephalopathy (TSE) cases in small ruminants. *The EFSA Journal* **276**, 1–30.

Eloit, M., Adjou, K., Coulpier, M., Fontaine, J. J., Hamel, R., Lilin, T., Messiaen, S., Andreoletti, O., Baron, T. & other authors (2005). BSE agent signatures in a goat. *Vet Rec* **156**, 523–524.

Europeancommunity (2001). Regulation (EC) No 999/2001 Of The European Parliament And Of The Council Of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Official Journal of the European Communities L 147/1.

Europeancommunity (2005). Commission Regulation (EC) No 36/2005 of 12 January 2005 amending Annexes III and X to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards epidemio-surveillance for transmissible spongiform encephalopathies in bovine, ovine and caprine animals. Official Journal of the European Union L 10/9.

Feraudet, C., Morel, N., Simon, S., Volland, H., Frobert, Y., Creminon, C., Vilette, D., Lehmann, S. & Grassi, J. (2005). Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrP^{Sc} replication in infected cells. *J Biol Chem* **280**, 11247–11258.

Foster, J. D. & Dickinson, A. G. (1988). The unusual properties of CH1641, a sheep-passaged isolate of scrapie. *Vet Rec* **123**, 5–8.

Fraser, H. & Dickinson, A. G. (1968). The sequential development of the brain lesion of scrapie in three strains of mice. *J Comp Pathol* **78**, 301–311.

Gavier-Widen, D., Noremark, M., Langeveld, J. P., Stack, M., Biacabe, A. G., Vulin, J., Chaplin, M., Richt, J. A., Jacobs, J. & other authors (2008). Bovine spongiform encephalopathy in Sweden: an H-type variant. *J Vet Diagn Invest* **20**, 2–10.

Goldmann, W., Hunter, N., Foster, J. D., Salbaum, J. M., Beyreuther, K. & Hope, J. (1990). Two alleles of a neural protein gene linked to scrapie in sheep. *Proc Natl Acad Sci U S A* **87**, 2476–2480.

Gretzschel, A., Buschmann, A., Eiden, M., Ziegler, U., Luhken, G., Erhardt, G. & Groschup, M. H. (2005). Strain typing of German transmissible spongiform encephalopathies field cases in small ruminants by biochemical methods. *J Vet Med B Infect Dis Vet Public Health* **52**, 55–63.

Gretzschel, A., Buschmann, A., Langeveld, J. & Groschup, M. H. (2006). Immunological characterization of abnormal prion protein from atypical scrapie cases in sheep using a panel of monoclonal antibodies. *J Gen Virol* **87**, 3715–3722.

Haraguchi, T., Fisher, S., Olofsson, S., Endo, T., Groth, D., Tarentino, A., Borchelt, D. R., Teplow, D., Hood, L. & other authors (1989). Asparagine-linked glycosylation of the scrapie and cellular prion proteins. *Arch Biochem Biophys* **274**, 1–13.

Harmeyer, S., Pfaff, E. & Groschup, M. H. (1998). Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. *J Gen Virol* **79**, 937–945.

Hill, A. F., Sidle, K. C., Joiner, S., Keyes, P., Martin, T. C., Dawson, M. & Collinge, J. (1998). Molecular screening of sheep for bovine spongiform encephalopathy. *Neurosci Lett* **255**, 159–162.

Hope, J., Wood, S. C., Birkett, C. R., Chong, A., Bruce, M. E., Cairns, D., Goldmann, W., Hunter, N. & Bostock, C. J. (1999). Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. *J Gen Virol* **80**, 1–4.

Ironside, J. W. (2010). Variant Creutzfeldt–Jakob disease. *Haemophilia* **16** (Suppl. 5), 175–180.

Jacobs, J. G., Langeveld, J. P., Biacabe, A. G., Acutis, P. L., Polak, M. P., Gavier-Widen, D., Buschmann, A., Caramelli, M., Casalone, C. & other authors (2007). Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe. *J Clin Microbiol* **45**, 1821–1829.

Jeffrey, M., Gonzalez, L., Chong, A., Foster, J., Goldmann, W., Hunter, N. & Martin, S. (2006a). Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunochemical similarities can be resolved by immunohistochemistry. *J Comp Pathol* **134**, 17–29.

Jeffrey, M., Martin, S., Gonzalez, L., Foster, J., Langeveld, J. P., van Zijderveld, F. G., Grassi, J. & Hunter, N. (2006b). Immunohistochemical features of PrP^{d} accumulation in natural and experimental goat transmissible spongiform encephalopathies. *J Comp Pathol* **134**, 171–181.

Klingeborn, M., Wik, L., Simonsson, M., Renstrom, L. H., Ottinger, T. & Linne, T. (2006). Characterization of proteinase K-resistant N- and C-terminally truncated PrP in Nor98 atypical scrapie. *J Gen Virol* **87**, 1751–1760.

Langeveld, J. P., Jacobs, J. G., Erkens, J. H., Bossers, A., van Zijderveld, F. G. & van Keulen, L. J. (2006). Rapid and discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. *BMC Vet Res* **2**, 19.

Le Dur, A., Beringue, V., Andreoletti, O., Reine, F., Lai, T. L., Baron, T., Bratberg, B., Vilotte, J. L., Sarradin, P. & other authors (2005). A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. *Proc Natl Acad Sci U S A* **102**, 16031–16036.

Lezmi, S., Martin, S., Simon, S., Comoy, E., Bencsik, A., Deslys, J. P., Grassi, J., Jeffrey, M. & Baron, T. (2004). Comparative molecular analysis of the abnormal prion protein in field scrapie cases and experimental bovine spongiform encephalopathy in sheep by use of Western blotting and immunohistochemical methods. *J Virol* **78**, 3654–3662.

Lezmi, S., Ronzon, F., Bencsik, A., Bedin, A., Calavas, D., Richard, Y., Simon, S., Grassi, J. & Baron, T. (2006). PrP^{d} accumulation in organs of ARQ/ARQ sheep experimentally infected with BSE by peripheral routes. *Acta Biochim Pol* **53**, 399–405.

Nicolas, O., Gavin, R. & del Rio, J. A. (2009). New insights into cellular prion protein (PrPc) functions: the ''ying and yang'' of a relevant protein. *Brain Res Rev* **61**, 170–184.

Nonno, R., Esposito, E., Vaccari, G., Conte, M., Marcon, S., Di Bari, M., Ligios, C., Di Guardo, G. & Agrimi, U. (2003). Molecular analysis of cases of Italian sheep scrapie and comparison with cases of bovine spongiform encephalopathy (BSE) and experimental BSE in sheep. *J Clin Microbiol* **41**, 4127–4133.

Notari, S., Capellari, S., Langeveld, J., Giese, A., Strammiello, R., Gambetti, P., Kretzschmar, H. A. & Parchi, P. (2007). A refined method for molecular typing reveals that co-occurrence of PrP^{Sc} types in Creutzfeldt–Jakob disease is not the rule. *Lab Invest* **87**, 1103–1112.

Oesch, B., Westaway, D., Walchli, M., McKinley, M. P., Kent, S. B., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B. & other authors (1985). A cellular gene encodes scrapie PrP 27–30 protein. *Cell* **40**, 735–746.

Polak, M. P., Zmudzinski, J. F., Jacobs, J. G. & Langeveld, J. P. (2008). Atypical status of bovine spongiform encephalopathy in Poland: a molecular typing study. *Arch Virol* **153**, 69–79.

Polak, M. P., Larska, M., Langeveld, J. P., Buschmann, A., Groschup, M. H. & Zmudzinski, J. F. (2010). Diagnosis of the first cases of scrapie in Poland. *Vet J* **186**, 47–52.

Prusiner, S. B. (1998). Prions. *Proc Natl Acad Sci U S A* **95**, 13363–13383.

Schaller, O., Fatzer, R., Stack, M., Clark, J., Cooley, W., Biffiger, K., Egli, S., Doherr, M., Vandevelde, M. & other authors (1999). Validation of a western immunoblotting procedure for bovine PrP^{Sc} detection and its use as a rapid surveillance method for the diagnosis of bovine spongiform encephalopathy (BSE). *Acta Neuropathol* **98**, 437–443.

Slootstra, J. W., Puijk, W. C., Ligtvoet, G. J., Langeveld, J. P. & Meloen, R. H. (1996). Structural aspects of antibody-antigen interaction revealed through small random peptide libraries. *Mol Divers* **1**, 87–96.

Somerville, R. A., Birkett, C. R., Farquhar, C. F., Hunter, N., Goldmann, W., Dornan, J., Grover, D., Hennion, R. M., Percy, C. & other authors (1997). Immunodetection of PrPSc in spleens of some scrapie-infected sheep but not BSE-infected cows. *J Gen Virol* **78**, 2389–2396.

Stack, M. J., Chaplin, M. J. & Clark, J. (2002). Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. *Acta Neuropathol* **104**, 279–286.

Tang, Y., Thorne, J., Whatling, K., Jacobs, J. G., Langeveld, J. & Sauer, M. J. (2010). A single step multiplex immunofluorometric assay for

differential diagnosis of BSE and scrapie. *J Immunol Methods* **356**, 29–38.

Thackray, A. M., Hopkins, L., Spiropoulos, J. & Bujdoso, R. (2008). Molecular and transmission characteristics of primary-passaged ovine scrapie isolates in conventional and ovine PrP transgenic mice. *J Virol* **82**, 11197–11207.

Thuring, C. M., Erkens, J. H., Jacobs, J. G., Bossers, A., Van Keulen, L. J., Garssen, G. J., Van Zijderveld, F. G., Ryder, S. J., Groschup, M. H. & other authors (2004). Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. *J Clin Microbiol* **42**, 972–980.

Thuring, C. M., van Keulen, L. J., Langeveld, J. P., Vromans, M. E., van Zijderveld, F. G. & Sweeney, T. (2005). Immunohistochemical distinction between preclinical bovine spongiform encephalopathy and scrapie infection in sheep. *J Comp Pathol* **132**, 59–69.

Weissmann, C. (2004). The state of the prion. *Nat Rev Microbiol* **2**, 861–871.

Wells, G. A., Scott, A. C., Johnson, C. T., Gunning, R. F., Hancock, R. D., Jeffrey, M., Dawson, M. & Bradley, R. (1987). A novel progressive spongiform encephalopathy in cattle. *Vet Rec* **121**, 419–420.

Wilesmith, J. W., Wells, G. A., Cranwell, M. P. & Ryan, J. B. (1988). Bovine spongiform encephalopathy: epidemiological studies. *Vet Rec* **123**, 638–644.