Sheep prions with molecular properties intermediate between classical scrapie, BSE and CH1641–scrapie

Jan PM Langeveld1,*, Jorg G Jacobs1, Jo HF Erkens1, Thierry Baron2, Olivier Andréoletti3, Takahashi Yokoyama4, Lucien JM van Keulen1, Fred G van Zijderveld5, Jim Hope6, Yue Tang6, and Alex Bossers1

1Department of Infection Biology; Central Veterinary Institute part of Wageningen UR; Lelystad, The Netherlands; 2Neurodegenerative Diseases Unit; ANSES-Lyon; Lyon, France; 3UMR INRA ENVT 1225; Interactions Hôtes Agents Pathogènes; Ecole Nationale Vétérinaire de Toulouse; Toulouse, France; 4National Institute of Animal Health; Tsukuba; Ibaraki, Japan; 5Department of Bacteriology and TSEs; Central Veterinary Institute part of Wageningen UR; Lelystad, The Netherlands; 6Animal Health and Veterinary Laboratories Agency; New Haw, Addlestone, Surrey, UK

Keywords: CH1641, prion, sheep, typing, Western blot

Abbreviations: AVG, average; BSE, bovine spongiform encephalopathy; IHC, immunohistochemistry; ic., intracerebrally; ip., intraperitoneally; PK, proteinase K; PrPc, prion protein in cellular form; PrPSc, proteinase K resistant fragment of PrPSc; PrPSc, prion protein in TSE associated form; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TE, tissue equivalents; triplex-WB, triplex Western blotting method; TSE, transmissible spongiform encephalopathy; VC, variation coefficient.

Efforts to differentiate bovine spongiform encephalopathy (BSE) from scrapie in prion infected sheep have resulted in effective methods to decide about the absence of BSE. In rare instances uncertainties remain due to assumptions that BSE, classical scrapie and CH1641—a rare scrapie variant—could occur as mixtures. In field samples including those from fallen stock, triplex Western blotting analyses of variations in the molecular properties of the proteinase K resistant part of the disease-associated form of prion protein (PrP\textsuperscript{res}) represents a powerful tool for quick discrimination purposes. In this study we examined 7 deviant ovine field cases of scrapie for some typical molecular aspects of PrP\textsuperscript{res} found in CH1641-scrapie, classical scrapie and BSE. One case was most close to scrapie with respect to molecular mass of its non-glycosylated fraction and N-terminally located 12B2-epitope content. Two cases were unlike classical scrapie but too weak to differentiate between BSE or CH1641. The other 4 cases appeared intermediate between scrapie and CH1641 with a reduced molecular mass and 12B2-epitope content, together with the characteristic presence of a second PrP\textsuperscript{res} population. The existence of these 2 PrP\textsuperscript{res} populations was further confirmed through deglycosylation by PNGaseF. The findings indicate that discriminatory diagnosis between classical scrapie, CH1641 and BSE can remain inconclusive with current biochemical methods. Whether such intermediate cases represent mixtures of TSE strains should be further investigated e.g. in bioassays with rodent lines that are varying in their susceptibility or other techniques suitable for strain typing.

Introduction

Transmissible spongiform encephalopathy’s (TSEs) are infectious neurodegenerative diseases with long incubation times and are diagnostically best characterized by depositions of prion protein (PrP) mainly in the central nervous system. In healthy conditions this protein is present as a cell membrane bound component (PrP\textsuperscript{c}) while in disease it is present as a refolded and aggregated product that is named PrP\textsuperscript{Sc}. Sc referring to scrapie \textsuperscript{-}, the archetypal prion disease condition occurring naturally in sheep and goats. Because of the peculiar nature of the infectious agent, composed only of protein, the term prion diseases has been generally used as an alternative term for TSEs after the introduction of the concept of the agent as prions.

In the 1980s, the emergence of an epidemic prion disease in cattle known as bovine spongiform encephalopathy (BSE) or mad cow disease has revealed the epizootic and zoonotic risks of...
TSEs. BSE not only affected at least 2 hundred thousand cattle mostly in the UK, but also has affected about 2 hundred people,\(^2\)\(^4\) cats,\(^5\) zoo-animals,\(^6\) and 2 goats.\(^7\)\(^9\) In humans the rise and decline of the disease known as variant Creutzfeldt-Jakob disease (vCJD) was epidemiologically linked to the BSE outbreak. Moreover, vCJD has strain properties similar to BSE both by typing in mice and by analyzing the molecular properties of its PrP\(^Sc\). The potential source of BSE is unknown but most probably has been a ruminant source either from a small ruminants scrapie variant or from a sporadic case of bovine TSE. The event for it to happen so recently was most likely an insufficiently safe process of rendering prion contaminated animal proteins into meat and bone meal.\(^10\)

The recognition and discrimination of different TSEs is an essential task. Diagnosis targeted on the N-terminal proteolytic processing of PrP\(^Sc\) is being an effective tool in discriminating TSE strains either without the use of protease digestion as in immunohistochemistry (IHC)\(^11\)\(^13\) or with additional digestion using proteinase K as performed in Western blotting, bead-based multiplex assays and ELISA assays.\(^14\)\(^18\) Of these techniques, Western blotting is able to both recognize PrP and provide essential information on different molecular PrP\(^Sc\) properties adding to the phenotypical palette of parameters for TSE strain typing. In this way, several types have been discovered in ruminants. In cattle 3 rather well recognized BSE variants have been defined being C-type, H-type and L-type BSE.\(^19\)\(^21\) Variant distinct TSE forms have been discriminated in sheep and goat by immunohistochemistry in tissue sections or Western blotting on PK digested brain homogenates such as classical scrapie, CH1641 scrapie, Nor98/atypical scrapie, and (experimental) BSE.\(^8\)\(^12\)\(^16\)\(^22\)\(^30\)

However, at least in small ruminants there are potentially several phenotypes present in the same host which pose basic questions about the nature of the agent, the source of the BSE epidemic and how to recognize the presence of BSE.\(^31\)\(^36\)

Recently we introduced 2 unique and powerful multiplex TSE differentiation non-enzymatic assays using fluorescence detection: one bead-based assay for TSE-typing in sheep\(^18\)\(^37\) and one probing technique for Western blots.\(^38\) These 2 techniques were effective to differentiate in brain homogenates between classical scrapie, CH1641, BSE, and Nor98/atypical scrapie in one test using 3 antibodies in a single mixture. The blotting technique, named triplex Western blotting (triplex-WB), is rather unique since the same protein (\textit{in casu} PrP) is simultaneously detected by 3 different primary PrP-specific antibodies on the same blotting membrane. The method eliminates potential errors in conventional Western blotting due to small migrational differences between gels as well as to variations in sample application when comparing different antibodies. Interestingly, the fluorescence detection method also is comparably sensitive to enzyme enhancement techniques and relatively quick with a low hands-on time.

In this study 7 French ovine field TSE cases were investigated by the triplex-WB technique.\(^38\) These cases were previously detected in France as deviating in conventional Western blots from the usual migration pattern of PrP\(^Sc\) of classical scrapie, and were categorized as CH1641-like in our bead-based methodology.\(^37\) By using a set of samples as reference for classical scrapie, CH1641 and BSE, the new triplex–WB data support the idea that especially in some of those deviant field cases variable levels of a second PrP\(^res\) population (also having a different N-terminal susceptibility to proteolytic cleavage) occur. The implications of the data indicate that biochemical diagnosis in brain homogenates alone is not always straightforward and that therefore other methods such as rodent bioassays for small ruminant scrapie typing remain essential.

### Results

The triplex-WB analyses on 7 deviant French TSE cases in this study (F1-F7, Table 1, Fig. 1) were intended to probe different structural properties of PrP\(^res\) by using antibodies 12B2, L42, and SAF84 that are specific for different PrP\(^Sc\) regions (Fig. 2). As references were used: ovine brain stem samples from sheep with confirmed classical scrapie (natural cases N1-N32 and experimental J1 distributed over 6 different PrP genotypes), experimental CH1641 (C1-C9 with genotypes ARQ/ARQ or AHQ/AHQ), and experimental BSE (B1-B4), respectively. Also one CH1641 infected goat case was included (G-gt). Where appropriate, bovine C-type (NL86) and H-type BSE (AU8) were used as additional controls.

### Protease susceptibility estimations of PrP\(^res\) N-terminus

Molecular mass of the non-glycosylated PrP\(^res\) moiety was probed in each sample with antibody L42. The variations in kDa values per individual scrapie sample were low, varying per sample (3 measurements per sample) with VCs between 1–8%. The molecular mass values in classical scrapie samples averaged at 21 kDa (average ± SD 21.2 ± 0.4 kDa, range 20.3-22.0), ovine CH1641 at 18.1 kDa (18.1 ± 0.9, range 17.7–19.0) and for ovine BSE at 18.3 kDa (18.3 ± 0.7, range 17.7-19.3) (Fig. 2A). These molecular mass values in the BSE and CH1641 cases differed significantly from those of the scrapie cases (\(P < 0.001\)). The molecular mass values in the study cases F1 and F3–F7 varied considerably between 17.7–19.7 kDa which is in the range of CH1641 and BSE references, while case F2 migrated as a 21.0 kDa protein, comparable to classical scrapie (Fig. 3A).

Next, the 12B2 epitope content relative to core epitope L42 was tested as a method for measuring the resistance of the PrP\(^res\) amino-terminus to PK processing under neutral pH conditions. The 12B2/L42 epitope ratios per individual scrapie sample varied with an average VC of 8% (range 1–24%). These ratios were similar between BSE and CH1641 isolates with values of respectively 0.04 ± 0.01 (range 0.02–0.05) and 0.09 ± 0.03 (range 0.06–0.12), and highly significantly higher in natural scrapie with values of 1.00 ± 0.25 (range 0.57–1.75, \(P < 0.001\)) (Fig. 3B). In the 7 F-cases, this parameter varied strongly with 12B2/L42 ratios ranging from 0.10–0.50, case F2 having the highest value. These 12B2/L42 values were clearly between on the one hand those of the natural scrapie and on the other hand of both CH1641 and BSE isolates.

Though the 2 parameters -molecular mass and relative 12B2 epitope content- are both related to cleavage of the PrP\(^res\)
| case | TSE type | status | route | species | PrP genotype | source |
|------|----------|--------|-------|---------|--------------|--------|
| F1/06-0287 | unusual, study cases | f.s. | natural | sheep | ARQ/ARQ | ANSES (FR) |
| F2/06-0412 | f.s. | natural | | | | |
| F3/PS048 | cl. | natural | | | | |
| F4/PS129 | cl. | natural | | | | |
| F5/PS1650 | cl. | natural | | | | |
| F6/PS227 | cl. | natural | | | | |
| F7/PS303 | cl. | natural | | | | |
| N1/0223685-0369 | classical scrapie | sl. | natural | sheep | ARH/VRQ | CVI (NL) |
| N2/96785103 | f.s. | natural | | | | |
| N3/60167-2861 | cl. | natural | | | | |
| N4/195777 | sl. | natural | | | | |
| N5/R07545 | f.s. | natural | | | | |
| N6/R09123-10 | f.s. | natural | | | | |
| N7/547189-4333 | cl. | natural | | | | |
| N8/612945-3921 | cl. | natural | | | | |
| N9/623789-3488 | cl. | natural | | | | |
| N10/12042 | sl. | natural | | | | |
| N11/38161 | sl. | natural | | | | |
| N12/38751-296 | f.s. | natural | | | | |
| N13/20824-0444 | f.s. | natural | | | | |
| N14/095773-0472 | f.s. | natural | | | | |
| N15/358279-1063 | f.s. | natural | | | | |
| N16/238243-0180 | sl. | natural | | | | |
| N17/390246-0732 | sl. | natural | | | | |
| N18/735007-0083 | sl. | natural | | | | |
| N19/2173083-1999 | f.s. | natural | | | | |
| N20/0060019-2171 | f.s. | natural | | | | |
| N21/R07090-6 | f.s. | natural | | | | |
| N22/36780-0763 | f.s. | natural | | | | |
| N23/1660717-6641 | f.s. | natural | | | | |
| N24/621553-4035 | cl. | natural | | | | |
| N25/R04768 | f.s. | natural | | | | |
| N26/R05096 | f.s. | natural | | | | |
| N27/123547-117 | f.s. | natural | | | | |
| N28/1256389-1156 | f.s. | natural | | | | |
| N29/R06925-11 | f.s. | natural | | | | |
| N30/465377-073 | sl. | natural | | | | |
| N31/2200994-0068 | f.s. | natural | | | | |
| N32/R07589-8 | f.s. | natural | | | | |
| J1/#73 | cl. | exptl | | | | |
| C1/#56 | CH1641 | cl. | exptl | sheep | ARQ/ARQ | NIAH (USA) |
| C2/PG1271/05 | cl. | exptl | | | | |
| C3/PG1275/05 | cl. | exptl | | | | |
| C4/PG1276/05 | cl. | exptl | | | | |
| C5/PG1283/05 | cl. | exptl | | | | |
| C6/PG0851/05 | cl. | exptl | | | | |
| C7/PG1284/05 | cl. | exptl | | | | |
| C8/8015470-6867 | cl. | exptl | | | | |
| C9/8015471-8173 | cl. | exptl | | | | |
| Cgt/8018337-1587 | cl. | exptl | goat | wt | CVI (NL) |
| B1/601955-8163 | BSE 1st pass. | cl. | exptl | sheep | wt | CVI (NL) |
| B2/6020175-8167 | cl. | exptl | | | | |
| B3/6019325-8024 | BSE 2nd pass. | cl. | exptl | | | |
| B4/6020305-8028 | cl. | exptl | | | | |
| NL86 | bo8SE-C | sl. | natural | cow | wt | CVI (NL) |
| AU8 | bo8SE-H | f.s. | natural | cow | wt | AGES (Austria) |

Notes. a The seven unusual TSE cases F1-F7 are the study cases which were diagnosed as CH1641-like in the bead-based assay. b Case numbers: first two or three characters in the numbers preceding the slash are referring to F, French study case; NL, classical scrapie from the Netherlands; J, Japan; C, CH1641-scrapie; B, BSE in sheep; NL86 and AU08, respectively bovine C-type BSE from The Netherlands and H-type BSE from Austria. Numbers after the slash are institutional source numbers. c TSE-type: cases B1-B2 and B3-B4 were derived respectively after 1st and 2nd passage in ARQ/ARQ sheep. d Status at collection: f.s., fallen stock; cl., clinical; sl. healthy at slaughter detected by active monitoring. e Route: exptl, experimental (C1-C7 ic.; C8, C9 and C-gt: ic.ip.). f Genotype: PrP genotype of sheep is usually described for both PrP alleles for the three codons 136 (A = alanine or V = valine), 154 (R = arginine or H = histidine), and 171 (Q = glutamine, R = arginine, or H = histidine), where the most common one is ARQ/ARQ (wild type); wt, wild type PrP genotype. g Source: supplying laboratory with between parentheses the original country source where the original isolate originated from.
N-terminus by PK, there was no obvious correlation between the 2 different measurements for these 7 F-samples (not shown). This discrepancy in these specific examples was highly probably due to variation in the amount of available ovPrP 93–97, 12B2–

Figure 1. Triplex-WB analysis picture of study cases together with some reference samples. Selection of samples analyzed include all study cases numbers F1–F7. All lanes contain ovine samples except lanes 10 and 22, respectively caprine CH1641 Cgt and bovine BSE-H. Lanes 6, 8, 9, 14, 19, and 21: ovine CH1641 samples C8, C9, C1, C7, C3, and C6; lane 11 experimental scrapie J1; lanes 23 and 24 respectively ovine classical scrapie N18 and BSE B4 (details of case codes in Table 1). In the triplex-WB a mix of the 3 antibodies L42, 12B2 and SAF84 was applied. As an example for the glycoprofile estimation, the white bar shows the typical triple band (3 arrows) area in the L42 and SAF84 channels, thus neglecting the 2 lower bands that are visible in CH1641 samples. The migration position of mono-glycosylated PrP^res fraction (at ± 24 kDa) is indicated with a black arrow, the di-glycosylated and non-glycosylated fractions (white arrows) migrate respectively above and below the 24kDa band in this triplet region. Marker proteins used are: rec-ovPrP in lane 1; molecular mass markers in lanes 2, 17, 18 and 25 with in lane 2 the molecular masses fl

Figure 2. Schematic bar diagram of PrP^res to illustrate the relative position of the 3 epitopes used in this study. The bar length reflects the length of a PrP monomer in classical scrapie PrP^res (PrP residues ± 76–234). The epitope position of antibodies 12B2, L42 and SAF84 are reflected by the colored boxes with respectively blue, red and green (color choices agree with wave length of fluorescence emission signals, see Methods section). The PrP location of antibodies 12B2, L42 and SAF84 corresponds respectively with that of group A, B and C antibodies as described by us previously.35 The location of the 2 glycosylgroups are indicated by S. The solid black lined box indicates the removal by proteinase K of N-terminal PrP region in BSE and CH1641, which in CH1641 is even a few residues further down stream than in BSE. The broken black lined box indicates the length of the second PrP^res population (PrP^res#2) as occurring in CH1641, where the 12B2 epitope is absent and the L42 epitope incomplete, thus only allowing full binding by SAF84. Sugar moieties are variably present in both PrP^res populations of CH1641, and in PrP^res from classical scrapie and BSE.

epitope over the 3 triplet bands. The estimation of the relative 12B2 epitope content becomes critical if protease cleavage occurs around amino acid tryptophan 9337,39 where presence or absence of this single amino acid will have an all or none effect on antibody 12B2 binding.40 Nevertheless, especially sample F2 is most likely a classical scrapie case with a sufficiently high amount of 21 kDa non-glycosyl band to yield a 12B2/L42 ratio of 0.5.

Protease susceptibility estimations of the PrP^res core

A method to test the protease susceptibility of the PrP^res core has previously been successfully used to discriminate between the bovine TSEs where C-BSE was found to be rather resistant compared to BSE type H.23 The method uses 2 PK digestion conditions—mild at pH 6.5 and stringent at pH 8, at respective PK concentrations of 50 and 500 µg.ml⁻¹ and a core specific antibody such as L42 as detecting antibody. The extent of resistance can be expressed as the ratio between L42 signal estimated at pH 8 and pH 6.5. Application to the ovine samples yielded susceptibility values for classical scrapie PrP^res ranging between 0.26–1.25 (0.72 ± 0.25), CH1641 between 0.11–0.89 (0.37 ± 0.25), and BSE as the most resistant between 0.82–1.16 (0.97 ± 0.17) (Fig. 3C). None of these differences however were statistically significant. The 7 F-cases varied between 0.42–1.50, like the variable behavior of the classical scrapie and CH1641 samples. Remarkably, 2 cases—F6 and F7—exhibited the highest values encountered in all sheep isolates of this study, being 1.31 and 1.50.

As a side note, it is a remarkable novel observation that the PrP^res-core PK resistance of the BSE samples is tending to be higher than that of classical scrapie, while the PrP^res N-terminal region of BSE is highly PK susceptible under mild conditions as was mentioned above for the 12B2/L42 ratios. The latter parameter nowadays being of critical discriminatory value in the diagnosis between BSE/CH1641 and classical scrapie.22,24

Glycosylation patterns

Glycosylation profiles of PrP^res are usually presented as the monoglycosylated (M) and diglycosylated (D) fraction in the
total triple band area, using a B-group antibody for detection i.e. L42. We employed the M/D ratio of these 2 glycosylated moieties for typing. The M/D ratio of BSE PrPres mono- and di-glycosyl fractions in the scrapie samples (0.49 ± 0.09) differed statistically (P < 0.05) from that of the set of CH1641 (0.42 ± 0.06) and BSE samples (0.33 ± 0.05) (Fig. 3D). None of the 7 study cases were outstanding in this respect, case F1 having an M/D ratio (0.54); the other cases F2-F7 ranged between 0.33–0.44.

Thus, consistent with other reports there is a tendency that ovine BSE cases contain a diglycosyl PrPres fraction around 65% (range 61–68%) of total PrPres which is higher than in scrapie cases (average 56%, range 47–67%), at the expense of mainly the mono-glycosylated moiety. The CH1641 cases contained a diglycosyl PrPres level similar to that in BSE (average 64%, range 62–70%), but mainly at the expense of the non-glycosylated moiety. This non-glycosylated PrPres fraction, was highest in scrapie cases (average 17%, range 11–23%), followed by BSE (average 14%, range 11–16%) and CH1641 (average 9%, range 7–11%).

**Investigating presence of a PrPres#2 population**

A feature of TSEs like CH1641 in sheep and H-type BSE in cattle is the presence of 2 populations of PrPres: population PrPres#1 exists as a triple band with molecular masses in the usual range between 17–32 kDa, and reactive with group B antibody L42 as well as group C antibody SAF84, while PrPres#2 also contains a triple band composition of lower molecular mass in the 10–25 kDa range but only reactive with the group C antibody SAF84. Evidence for the existence of the #2 population can be deduced from the ratio of SAF84/L42 signal in the fraction of mono-glycosylated PrPres (or 24kDa) region within the triple band region of the L42 reactive bands. In the sheep derived samples all experimental CH1641 samples exhibited such SAF84/L42 ratios between 1.4-2.0, and 5 of the 7 field samples under study–F1, F3, F4, F5 and F7 – yielded ratios of 1.2 or higher, indicative for presence of a certain amount of PrPres#2 population (Fig. 3E). Both the 3 two classical scrapie reference samples, the experimental classical scrapie J1 and the 4 experimental BSE cases appeared to contain only one PrPres population based on ratio values ranging between 0.7-1.1. The variation in

**Figure 3.** Western blot results transformed into values to express 5 different PrPres properties. In the 5 panels are expressed: (A) molecular mass of non-glycosylated PrP as observed with mAb L42 (see Fig. 1); (B) N-terminal epitope content expressed as 12B2/L42 ratio; (C) PK resistance as a ratio of L42 signals at pH 8 and pH 6.5; (D) glycoprofile expressed as L42 signal ratio M/D, where M and D are the respective mono-glycosylated and di-glycosylated fractions of the 3 PrPres bands (see Fig. 1); (E) marker for the presence of a dual PrPres population as estimated by SAF84/L42 ratio of the 24 kDa fraction of total PrPres triplet (see Fig. 1). Black bars represent averages ± SD of the reference samples indicated on the horizontal axis with the number of different samples per group between parentheses. The open bars represent data of the 7 study samples F1–F7 with averages ± SD for 2–7 measurements per sample, except for panel c where single measurements have been carried out; for these cases bar-values are specified above the bars.
the SAF84/L42 ratios at the 24kDa band for these samples were low at an average VC of 10 ± 6%, indicative for a reliable way of estimating this dual population parameter.

To find more support for this dual population property, deglycosylations were carried out with PNGaseF. As expected for CH1641 (and H-type BSE), this treatment yielded 2 deglycosylated PrPres bands, of which band 1 migrated at the usual 17-21 kDa position, and band 2 at the CH1641 specific position 10-13 kDa (Fig. 4A). All CH1641 samples yielded a dual PrPres population result by the presence of band2 (band2/band1 ratios > 0.2). In the classical scrapie samples investigated band 2 was hardly or not present, with values between 0.05 and 0.15 while reference cases N7 and N22 (and B1 as well) had values close to 0.2. Four study cases—F1, F2, F5 and F7—yielded values > 0.20, but not F3 while cases F4 and F6 were too weak for analysis. Finally, when the SAF84/L42 ratio of all samples analyzed for both parameters was plotted against band2/band1 ratio for linear regression analysis, it appeared that there was a positive correlation between these 2 variables ($R^2 = 0.74$), though study samples F2 and F3 did poorly fit this correlation (Fig. 4B).

Combining the observations

To classify the TSE type in the 7 French field sheep cases for their PrPres features by Western blotting, we used confirmed cases of classical scrapie cases and experimentally generated infected BSE– and CH1641–brain tissues from sheep as reference. Within these reference groups, the 3 most crucial and discriminating molecular PrPres parameters were the molecular mass of the non-glycosylated moiety (kDa), the relative presence of the 12B2 N–terminus epitope (12B2/L42 signal ratio in the triple band area), and the occurrence of a second population (indicated by the SAF84/L42 signal ratio of the 24kDa band fraction). The 7 study samples exhibited some CH1641 property (cases F1-F3, F5, and F7), or similarities with classical scrapie (F6) or BSE (F4), the latter 2 both weak cases for which a band 2 estimation was not possible; however neither of these 2 did fit completely a diagnosis for classical scrapie, CH1641 or BSE (Fig. 5). Other references included: an experimentally generated goat CH1641 sample (Cgt) which behaved similar to the experimental sheep CH1641 cases C2-C8 (inoculum originally sourced from the same UK source); experimentally generated sheep scrapie (J1) and CH1641 (C1) of which both were subpassaged in sheep from the same USA classical scrapie infected animal and which behaved similarly respectively to Dutch natural scrapie and experimentally generated UK sourced CH1641; and bovine C- and H-type BSE samples included to compare with ovine BSE and CH1641 (Fig. 5). Furthermore, there were no statistically significant differences for any of the 5 parameters discussed above between the 6 different sheep genotypes with classical scrapie. Bovine and ovine BSE were very comparable in all parameters.
estimated. Bovine H-type BSE and ovine CH1641 did show presence of a dual PrPres population but they differed in the relative content of the 12B2 N-terminus epitope being amply present in H-type, but close to zero in CH1641.

**Discussion**

The molecular analyses by triplex-Western blotting in this study of 7 ovine field TSE cases from France confirmed their separate status with respect to the features that usually allow a diagnosis of classical scrapie, BSE and CH1641 in sheep. Whether these isolates represent separate scrapie strains or mixtures of different TSE strains remained undetermined, but either possibility might well be the case. Nevertheless, the data obtained here point to TSE heterogeneity, the quasi-species nature of sheep prions or the existence of different strains in field TSEs of sheep. The strongest features for type recognition in Western blots appear to be 3 parameters: the molecular mass of the non-glycosylated PrPres moiety, the estimation of the amounts of (ovine) PrP epitope 93–97 (12B2) in relation to the core 148–153 epitope (L42), and the presence of a second PrPres population (PrPres#2) that is typical for CH1641 and well recognized only by group C antibodies (e.g., SAF84) reactive with an epitope C-terminal of the ovine PrP amino acid position 154. The 2 other parameters—PrPres glycoprofile and PK susceptibility of the PrPres core estimated by using a group B antibody—were too variable within the reference TSE types to allow a firm recognition.

The results of the Western blotting technique, even though not fulfilling the expectation to clearly recognize these 7 sheep TSE cases as classical scrapie, BSE or CH1641, do confirm the power of the 2 discriminative parameters defined in our previous study: N-terminal processing of PrPres by PK and presence of 2 PrPres populations. The blotting system allowed to deduce these 2 aspects with a selected triplex antibody usage applied on the same protein in one single blot leading to low interassay variations. Variations in proteolytic processing of the PrPSc N-terminus can be expressed as molecular mass (kDa) or the ratio of the immuno-signals between e.g. 12B2 and L42, while the presence of 2 PrPres populations is evident by the SAF84/L42 ratio in the 24 kDa fraction of the PrPres triple band region. Glycoprofile estimation of BSE cases estimated with a group B antibody (like L42, 6H4, Sha31) is another aspect characterized by a high diglycosyl fraction, while indeed was the case varying between 60–70% of total PrPres, while that in the natural scrapie cases varied between 47–67% and CH1641 between 62–70%. However, this overlapping result in diglycosyl PrPres concentration between the 3 sheep TSE types makes it an indicative but inadequate marker for a distinct typing diagnosis.

The existence of a separate TSE status of the study cases does not seem genotype or tissue quality dependent since one of the 2 fallen stock ARQ/ARQ samples (F1) and several of the VRQ/VRQ freshly collected samples (F3, F4, F5, F7) could be categorized as cases with a dual PrPres population. Moreover, extra N-terminal PrPres processing by autolysis or microbial breakdown would have shown up in many more instances of European monitoring or in the fallen stock classical scrapie references of this study. Also, PrPres properties might be influenced by the brain region chosen for analysis. A difference in outcome of a sheep was suggested where a scrapie as well as a BSE diagnosis had been obtained by using different techniques in probably different laboratories in respectively the caudal medulla and the brain stem. The chance that our observations on PrPres properties might be dependent on the region in the brain cannot be ruled out, but seems negligible since in all cases homogenized brain stem material was used.

In a previous study using a bead-based multiplex immunoassay for TSE type discrimination, we diagnosed all 7 French cases as CH1641-like, while in the current triplex-WB analysis a CH1641-like diagnosis was applicable to cases F1, F3, F5, and F7, maybe to F2 but not to weak cases F4 and F6. In that study the binding of the group C antibody 94B4 was essential for capture to a bead of both the PrPres#1 and PrPres#2 population, while antibody Sha31—a group B antibody very similar in epitope specificity to L42 by epitope mapping—was used as a reporter for bead-captured PrPres. However, it should be realized that the 2 methods have really different principles. The triplex-WB yields a more detailed result due to the fact that binding of all 3 antibodies is observed on one single molecule while in the bead-based assay only one antibody can bind per PrP molecule. Therefore, the bead-based multiplex assay remains a remarkable test system with unique structural resolution, different from Western blots and ELISAs.

The molecular properties of the 7 cases with their PrPres properties intermediate between BSE, classical scrapie and CH1641 pose a basic question about the nature of prions: represent these cases separate strains, or mixtures of different strains, or are prions appearing as quasi-species where the fittest dominates under a certain biological condition? These are questions that would need further investigations. The existence of mixtures of scrapie types in sheep has been discussed since the time that genetic susceptibility for TSEs has been described using CH1641 and SSBP/1 as experimental challenge material. Furthermore, for 4 French ovine CH1641-like cases with a low P4 (with nearly similar PrP binding location as 12B2) epitope content in PrPres it was shown in transgenic TgOPrP mice expressing ovine ARQ PrP that indeed a CH1641 feature was transmitted characterized by the presence of the 2 PrPres populations PrPres#1 and PrPres#2 characteristic for experimental CH1641. However, the presence of a typical classical scrapie feature was not shown in these mice, for which reasons the presence of a mixture of classical scrapie and CH1641 remains uncertain. In our study, the 7 cases variably exhibited a 12B2 epitope content intermediate between that found in classical scrapie and reference ovine BSE cases; of these 7 (F1, F3, F5 and F7) exhibited evidence for the presence of a PrPres#2 population as in CH1641-like isolates, while 2 cases (F2, F6) were close to classical scrapie and one (F4) most close to BSE. However cases F4 and F6 were rather low in PrPres content limiting the reliability of the detection and hampering an enzymatic deglycosylation analysis with PNGaseF. Low levels of PrPres#2 in naturally TSE infected
sheep brain samples seem anyway to be sometimes present in sheep with classical scrapie\textsuperscript{26} and this also might be the case in some of our sheep reference samples. Nevertheless Western blot analyses only are not sufficient to understand the nature of sheep prions, but maybe these cases could serve as interesting targets for further research.

The existence of deviant field TSE cases in sheep as found in this study and others based on PrP\textsubscript{res} properties\textsuperscript{16,26} call for further investigation. Bioassays including limited titration experiments in rodents and transgenic mice, or even other techniques such as in vitro conversion studies or permissive TSE-susceptible cell systems are necessary to get additional confirmation of strain typical features. This will not only be required for TSEs in sheep but also in goats which are also susceptible to BSE and different scrapie forms as found in sheep.

\section*{Materials and Methods}

\textbf{Materials}

To serve molecular TSE typing 3 antigenic PrP regions A, B and C have been defined recognizing respective epitope regions A, B and C which are located from N- to C-terminus in the respective ovine PrP sequence positions 60–100, 101–154 and 154–234 (Fig. 2).\textsuperscript{35} Purified monoclonal PrP-specific antibodies used (with their antigenic group specificity, Ig class type, and source) were: 12B2 (group A, IgG1, CVI-WageningenUR), L42 (group B, IgG2a, R-Biopharm), and SAF84 (group C, IgG2b, SpiBio, Bertin Pharma).\textsuperscript{40,47-49} The respective epitope specificities of 12B2, L42 and SAF84 were \textsuperscript{12G}W\textsuperscript{148}ED\textsuperscript{153}RY\textsubscript{154}Y1 for (sheep PrP numbering, glutamine on position 171)\textsuperscript{50} as mapped by Pepscan analysis using solid phase synthetic peptides based on the ovine PrP sequence.\textsuperscript{51} Mouse IgG class-specific detection kits containing isotype-specific goat anti-mouse-Fc Fab-Alexa Fluor 488, 555, 647 (sheep PrP numbering, glutamine on position 171)\textsuperscript{50} and 0.5 \textmu m filter. 12B2, SAF84 and L42 conjugates were purchased as Zenon\textsuperscript{®} labels (Life Technologies). Proteinase K was from Merck (30 U/mg; 124568; Darmstadt, Germany). Purified recombinant ovine PrP\textsubscript{ARQ} was a kind gift from Human Rezaei, (INRA, Jouy-Josas). Molecule mass markers kit See Blue was from Life Technologies.

\textbf{Tissues}

Fifty 6 different brain stem samples have been investigated, nearly all from sheep except one goat sample and 2 bovine BSE cases. These tissues are collectively detailed in Table 1 including the supplying institute and the country from where the tissue was originating from. Also PrP genotypes of the individual sheep samples are explained in Table 1.

Seven deviant scrapie cases from different flocks (F1-F7) were investigated in this study because of unusual behavior in our head-based assays\textsuperscript{52}: F1 and F2, both of PrP genotype ARQ/ARQ, were collected from fallen stock monitoring, and F3-F5 (all VRQ/VRQ) from clinical surveillance.

As references in this study, CH1641 samples were obtained from 5 institutes to cover the places world wide where CH1641 materials have been discovered or generated. Three different sheep TSE types were used: 3two natural classical scrapie cases (N1-N32) from active and passive surveillance and varying in PrP genotype, 8 CH1641 experimentally infected samples from sheep (C2-C9) and one goat (Cgt) challenged with CH1641/3 which is a UK derived brain pool from 5 SIP\textsuperscript{6/}ps sheep (presumed PrP genotype ARQ/ARQ) and kindly provided by Roslin Institute, (University of Edinburgh, UK),\textsuperscript{32} 4 experimentally BSE infected samples after 1st (B1, B2) or 2nd (B3, B4) passage. In addition included were brain stems from 2 different Cheviot sheep i.e. infected with brain from either experimental classical scrapie case #2314 (J1) or experimental CH1641 case #294 (C1); the original scrapie isolate used at 1st passage in both sheep #2314 and #294 was a classical scrapie isolate form the USA.\textsuperscript{36} Furthermore, 2 bovine cases of different BSE type have also been used, one of which was a case of C-type (NL86) and the other of H-type BSE (AU8).\textsuperscript{32} Confirmed TSE-negative brain stem tissues from sheep served as negative controls. All cases were confirmed by immuno-histochemistry and/or Western blotting.

\textbf{PrP\textsubscript{res} preparation and triple Western (triplex-WB) blotting analyses}

Preparation of 10 per cent tissue homogenates in lysis buffer, conditions for digestion with proteinase K (PK) to prepare PrP\textsubscript{res} from PrP\textsubscript{Sc}, precipitation of PrP\textsubscript{res} with 1-propanol, sodium dodecyl sulfate PAGE (SDS-PAGE), subsequent triplex-WB and fluorometric detection were as described\textsuperscript{38} with the following details. Blots were developed with a mixture of the antibodies 12B2, L42, and SAF84 at respective Alexa Fluor labels 488, 555, 647 (blue, green and red in Fig. 2). Blots were dried in a vacuum desiccator for 1 hour at ambient temperature. Molecular masses of triplet PrP\textsubscript{res} bands detected with L42 were calculated using GelPro software (Get-Pro analyzer; Media Cybernetics) and with InvitroGen SeeBlue\textsuperscript{®} prestained molecular mass markers (Life Sciences) as applied in 2 lanes per gel; these standards have a high fluorescence yield in the 488 nm channel. In all runs rec-ovPrP\textsubscript{ARQ} was included as a correction reference for signal level differences between emission light intensities in the 3 different Typhoon Trio laser light channels. As a further correction to quantify the relative N-terminal 93–97 epitope presence in 12B2/L42 ratio estimations, the average 12B2/L42 value for all scrapie samples in an experiment (usually n = 32) was considered to be 1,\textsuperscript{39} therefore each individual 12B2/L42 outcome was multiplied with 1/(12B2\textsubscript{scr}/L42\textsubscript{scr}) where the 12B2\textsubscript{scr}/L42\textsubscript{scr} ratio is representing the average 12B2/L42 value of all 3two classical scrapie cases in the experiment. Glycoprofiles were estimated as the percentual fraction of di-, mono- and non-glycosylated PrP\textsubscript{res} in the triple band area.\textsuperscript{38} For estimating the presence of a second population of PrP\textsubscript{res}, the L42 and SAF84 signals in the monoglycosylated PrP\textsubscript{res} band (migrating at 24-25 kDa) expressed as fraction of the total PrP\textsubscript{res}-triplet signal were used to calculate the SAF84/L42 ratio; a value > 1.2 points
to the presence of a significant amount of PrPSc#2 population that is typical for CH1641-like cases.

**Differential PK digestions to test protease susceptibility of PrPSc core**

Determination of the resistance of the PrPSc core to PK was performed as described21 by comparing samples digested at pH 6.5 and 8 and respective enzyme concentrations 50 μg ml⁻¹ 500 μg ml⁻¹ PK. Triplex-WB was used with antibody L42 as PrPSc core region detecting antibody. The relative breakdown of the PrPSc core was expressed as the pH8/pH6.5 signal ratio between each set of lanes probed in the triple band region, where values between zero and one related to respectively high and low PK susceptibility.

**Deglycosylation of PrPSc by treatment with PNGase F**

Deglycosylation of PrPSc preparations with peptide N-glycosidase F (PNGase F; New England Biolabs) was performed as described previously.40

**Statistical analyses**

Interassay variation for a sample value was expressed as variation coefficient (VC) being the percentage of standard deviation (SD) of the average value (AVG) of a group of values found for that sample, thus VC = (SD/AVG)×100%. One-way analyses of variance were carried out to establish whether variations between groups of data were larger than expected; if so, subsequent differences between pairs of groups were considered significant if the probability (P) of a non-difference was <0.05 in multiple-comparisons tests, according to the Student-Newman-Keuls test. The software used for these calculations was with Prism 5.02 software from Graph-Pad Software.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Dr. Jim Foster from Roslin institutes (UEDIN, Edinburgh, UK) for supply of CH1641 challenge material used for generating the different experimental CH1641 tissues at CVI and AHVLA. Dr. Jacques Grassi (CEA, Sacy, France) and Dr. Martin Groschup (FLI, Riems-Greifswald, Germany) provided kindly an initial batch of respectively antibodies SAF84 and L42. We thank Dr. Human Rezaei (INRA, Jouy-en-Josas, France) for kind gift of rec-ovine PrPARG.

**Funding**

This work was largely supported by the Dutch Ministry of Economic Affairs (grant WOT-01-002-01). Part of this work was also possible thanks to Department for Environment, Food and Rural Affairs (Defra, UK; project SE1700/CSA 7335), European Commission funded STREP project GoatBSE, FOOD-CT-2006-36353 and EMIDA ERA-NET project GOAT-TSE-FREE.

### References

1. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. Science 1982; 216:136-44; PMID:6801762; http://dx.doi.org/10.1126/science.6801762
2. Wells GA, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, Dawson M, Bradley R. A novel progressive spongiform encephalopathy in cattle. Vet Rec 1987; 121:419-20; PMID:3426065; http://dx.doi.org/10.1136/vr.121.18.419
3. Will RG, Ironside JW, Zeidler M, Causes SS, Esti-beiro K, Alpenovich A, Poser S, Pocchiari M, Hofman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996; 347:921-5; PMID:8598754; http://dx.doi.org/10.1016/S0140-6756(96)91412-9
4. Bruce ME, Boyle A, Causes S, McConnell I, Foster J, Goldmann WG, Fraser H. Strain characterization of natural sheep scrapie and comparison with BSE. J Gen Virol 2002; 83:695-704; PMID:11842264
5. Wyard JM, Pearson GR, Smardon TN, Grefffe-Jones TJ, Wells GA, Wilesmith JW. Naturally occurring scrapie-like spongiform encephalopathy in five domestic cats. Vet Rec 1991; 129:233-6; PMID:1957458; http://dx.doi.org/10.1136/vr.129.11.233
6. Patinson J. The emergence of bovine spongiform encephalopathy and related diseases. Emerg Infect Dis 1998; 4:390-4; PMID:9716952; http://dx.doi.org/10.3201/eid0403.980311
7. Eloit M, Adou K, Coupliez M, Fontaine JJ, Hamel R, Lilin T, Messiaen S, Andreolleti O, Baron T, Benczik A, et al. BSE agent signatures in a goat. Vet Rec 2005; 156:523-4; PMID:15833975; http://dx.doi.org/10.1136/vr.156.16.523-b
8. Jeffrey M, Martin S, Gonzalez L, Foster J, Langeveld JP, van Zijderveld FG, Grassl J, Hunter N. Immunohistochemical features of PrP(d) accumulation in natural and experimental goat transmissible spongiform encephalopathies. J Comp Pathol 2006; 134:171-81; PMID:1654272; http://dx.doi.org/10.1016/j.jcpath.2005.10.005
9. Spiropoulos J, Locey R, Sallis RE, Terry LA, Thorne L, Holder TM, Beck KE, Simmons MM. Isolation of prion with BSE properties from farmed goat. Emer Infect Dis 2011; 17:2253-61; PMID:22172120; http://dx.doi.org/10.3201/eid1712.110333
10. Wilesmith JW, Ryan JB, Atkinson MJ. Bovine spongiform encephalopathy: epidemiological studies on the origin. Vet Rec 1991; 128:199-203; PMID:1823120; http://dx.doi.org/10.1136/vr.128.9.199
11. Jeffrey M, Martin S, Gonzalez L, Ryder SJ, Bellworthy SJ, Jackman R. Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. J Comp Pathol 2001; 125:271-84; PMID:11798244; http://dx.doi.org/10.1053/jcpath.2001.0499
12. Thuring CM, van Keulen LJ, Langeveld JP, Vromans ME, van Zijderveld FG, Sweeney T. Immunohistochemical distinction between preclinical bovine spongiform encephalopathy and scrapie infection in sheep. J Comp Pathol 2001; 125:271-84; PMID:11798244; http://dx.doi.org/10.1053/jcpath.2001.0499
13. Jeffrey M, Gonzalez L, Chong A, Foster J, Goldmann W, Hunter N, Martin S. Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunohistochemical similarities can be resolved by immunohistochemistry. J Comp Pathol 2006; 134:177-29; PMID:16324707; http://dx.doi.org/10.1016/j.jcpath.2005.06.005
14. Baron TG, Mader JY, Calavas D, Richard Y, Barrillot F. Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. Neurosci Lett 2000; 284:175-8; PMID:10776427; http://dx.doi.org/10.1016/S0304-3940(00)00147-4
15. Hill AF, Sidle KC, Joiner S, Keyes P, Martin TC, Dawson M, Collinge J. Molecular screening of sheep for bovine spongiform encephalopathy. Neurosci Lett 1998; 255:159-62; PMID:9832197; http://dx.doi.org/10.1016/S0304-3940(98)00073-6
16. Hope J, Wood SC, Birkett CR, Chong A, Bruce ME, Cairns D, Collinge J. Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. J Gen Virol 1999; 80 (Pt 1):1-4; PMID:9934675
17. Simon S, Nugier J, Morel N, Boutil H, Creminon C, Benestad SL, Andréolleti O, Lantier F, Bilheume JD, Fessyguet M, et al. Rapid typing of transmissible spongiform encephalopathy strains with differential ELISA. Emer Infect Dis 2008; 14:686-16; PMID:18394279; http://dx.doi.org/10.3201/eid0404.071134
18. Tang Y, Thorne J, Whatling K, Jacobs JG, Langeveld J, Saus ME. A single step multiplex immunofluorometric assay for differential diagnosis of BSE and scrapie. J Immunol Methods 2010; 356:29-38; PMID:20214905; http://dx.doi.org/10.1016/j.jim.2010.03.002
19. Basabe AG, Laplanche JL, Ryder S, Baron T. Distinct molecular phenotypes in bovine prion diseases. EMBO Rep 2004; 5:110-5; PMID:14710195; http://dx.doi.org/10.1038/sj.embor.7400054
20. Casalone C, Zanussi G, Acutis P, Ferrari S, Capacci L, Taglialin F, Monaco S, Caramelli M. Identification of...
