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Four Independent Molecular Prion Protein Parameters for Discriminating New Cases of C, L, and H Bovine Spongiform Encephalopathy in Cattle▽

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In anticipation of the emergence of more variants of bovine spongiform encephalopathy (BSE), a semiquantitative display of the following four independent molecular diagnostic prion parameters was designed: N terminus, proteinase K (PK) resistance, glycoprofile, and mixed population. One H BSE case, three L BSE cases, six C BSE cases, and one unusual classical BSE (C BSE) case are reported.

The epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom and other countries, with the subsequent emergence of a variant form of Creutzfeldt-Jakob disease in humans, had urged the European Union (EU) in 2001 to take costly measures by active monitoring cattle for BSE and banning the recycling of mammalian offal proteins into feed (8). Eight years later, due to the effectiveness of the control measures taken, new BSE cases rarely occur.

It has become evident that atypical BSE types exist in cattle (2, 5). These variants seem to be sporadic, and all occurred in animals 8 years and older (2–7, 10, 13–15, 17–19, 1–23), except one isolated 2-year-old case (24). BSE cases are now classified into classical BSE (C BSE), L BSE, and H BSE based on Western blot (WB) analysis of PrP^{res}, the proteinase K (PK)-resistant fragment of the disease-associated prion protein (PrP) isoform PrP^{Sc} (15). It must be anticipated that with continuing active surveillance, a majority of cases will appear as natural sporadic forms (2). Confirmation of such cases requires effective criteria.

In this study, the molecular properties of brain stem PrP^{res} in eight Austrian and three recently reported BSE cases in the Netherlands (Table 1) were investigated by WB analysis with PrP-specific monoclonal antibodies (MAbs) 12B2, P4, L42, and SAF84. These are specific for bovine PrP epitopes 101WGQGG105, 101WGQGGSH107, 156YEDRYY161, and 174YRPVDQY180, respectively (1, 2, 9, 11, 12, 14, 20). Two WB detection methods were applied. The first method used was based on enzymatic chemiluminescent enhancement procedures followed by photographic film exposure (15), and the second WB method used was a recently developed multiplex fluorophore labeling method and was used here for glycoprofile estimations (16). This multiplex technique provided a reliable tool for glycotyping for two reasons: the absence of substrate spreading from the protein banding regions and the area of measurement is exactly the same when comparing the PrP^{res} glycoprofiles for different MAbs. Relative antibody staining intensities between blots, between two lanes, or for PrP^{res} glycoform estimations were performed as described previously (15, 16).

Western blot PrP^{res} patterns obtained for the samples were similar to those reported for bovine C, L, and H BSE cases (15, 16). These patterns of MAb binding were transformed semiquantitatively as ratios of staining intensities reflecting four independent PrP^{res} aspects in two graphs (Fig. 1a and b). The first aspect concerned the N-terminal aspect, represented by the 12B2 epitope. Most BSE samples had small amounts of this epitope (12B2/L42 ratio of <0.4), typical for C and L BSE. Sample AU08 exhibited relatively high N terminus epitope retention, as did the H BSE reference sample (Fig. 1a, vertical axis). In addition, both AU08 and the reference H sample contained an additional 7-kDa band with antibodies 12B2 and L42 (Fig. 2) (15). One Austrian case (AU05) was special since it reacted with substantial 12B2 intensity compared to MAb L42 (Fig. 2, lanes 1). Moreover, when probed with N terminus-specific MAb P4 (epitope WGQGGSH, which carries the same N-terminal amino acid as the epitope of 12B2), there was unusually high binding of this antibody to AU05, about equal to that of MAb 12B2, not seen in any of the N terminus epitope-carrying H BSE cases (Fig. 2, compare lanes 1 and 2) or in our previously studied C BSE cases (15, 16).

A further tool for diagnosis was the analysis of resistance to PK digestion by comparing samples digested at pH 6.5 with 50 μg/ml PK and at pH 8 with 500 μg/ml PK (Fig. 1b, vertical axis). Samples AU01 to AU05 and NL86 and NL87 appeared equally resistant to PK as did the reference C BSE samples, reflected by pH 8/pH 6.5 ratios of between 0.7 and 1. Samples AU06, AU07, AU08, and NL88 compared to the poorly PK-resistant L and H BSE reference samples.

PrP^{res} is usually characterized by a triple banding pattern that is composed of nonglycosylated (N), monoglycosylated (M), and diglycosylated (D) PrP fragments having molecular masses around 18, 22, and 28 kDa, respectively (Fig. 2). The sum of the antibody binding values for N+M+D fragments in this region was set at 1, and their fragment fractions were used for ratio calculations shown in Fig. 1a and b. When tested with

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TABLE 1. Details of Austrian and Dutch BSE cases until March 2011[a]

Case no.	Yr of birth	Age (mo)	Screening test[b]	Confirmation test[c]	Status at death	Typing result[d]
AU01	1996	70	PCW	OIE-WB	Healthy	C (A1B2)
AU02	1994	134	TeSeE	OIE-WB	Healthy	C (A1B2)
AU03	1992	155	LIA	IHC	Healthy	C (A1B2)
AU04	2000	70	LIA	IHC	Healthy	C (A1B2)
AU05	1993	149	LIA	OIE-WB	Fallen st.	C^{P4} (A2B2)
AU06	1996	130	LIA	IHC	Fallen st.	L (A4B1)
AU07	1997	150	PCP	Bio-Rad-WB	Healthy	L (A4B1)
AU08	1995	180	PCP	Bio-Rad-WB	Fallen st.	H (A2B4)
NL73	1996	91	PCW	IHC	Healthy	C (A1B2)
NL74	1996	90	PCW	IHC	Healthy	C (A1B2)
NL86	1999	140	PS	IHC	Healthy	C (A1B2)
NL87	1997	162	PCW	IHC	Fallen st.	C (A1B2)
NL88	1996	174	PCW	IHC	Fallen st.	L (A4B1)

[a] All Austrian cases were investigated for the type of BSE for the first time, as well as the most recent Dutch cases (NL86 to -88), plus some confirmed C BSE cases from a previous study (NL73, NL74) in which all Dutch cases had been analyzed (15). Fallen st., fallen stock (i.e., animals that died on the farm).

[b] PCW = Prionics-Check Western blot; TeSeE = enzyme-linked immunosorbent assay (ELISA)-like Bio-Rad TeSeE test; LIA = ELISA-like Prionics-Check luminescence immunoassay; PCP = immunochromatographic lateral diffusion Prionics-Check PrioSTRIP test; PS = Roche PrionScreen ELISA-like test.

[c] OIE-WB = World Organisation for Animal Health (OIE)-recognized methods using scrapie-associated fibril (SAF) purification and Western blot detection; IHC = OIE-recognized method using immunohistochemistry; Bio-Rad-WB = an EU-recognized PrP^{res} preparation method, followed by Western blotting.

[d] Typing results (and classifications, shown in parentheses) were derived from Fig. 1. AU05 is a C-type BSE case with intermediately high MAb 12B2 reactivity and unusually high MAb P4 binding comparable to that of 12B2. Such high P4 affinity has not been observed previously, neither for intact bovine PrP nor its fragments (Fig. 2).

MAb L42, a low M-to-D fragment ratio (≤0.3) was observed for the samples AU01, AU02, AU03, AU04, AU05, NL73, NL74, NL86, and NL87 as well as for the two reference C BSE samples (Fig. 1a, horizontal axis). Samples AU06, AU07, NL88, and reference L BSE exhibited a higher fraction of M-PrP^{res} (M/D fragment ratio = ±1). Compared to the C and L BSE-like cases, the AU08 sample and the reference H BSE M/D fragment values were intermediate (M/D fragment ratio = ±0.5).

As a fourth criterion, glycoprofile results between those obtained with MAbs L42 and SAF84 were compared, and they were rather similar for most samples, except for case AU08 and the reference H BSE sample. This could be illustrated in the plot by the binding ratio between SAF84 and L42 at the M position (Fig. 1b, horizontal axis). This so-called dualistic behavior was previously shown to be a result of the existence of a mixture of two PrP^{res} populations in H BSE isolates (1, 15).

When dissecting the graphs shown in Fig. 1 into four sectors,

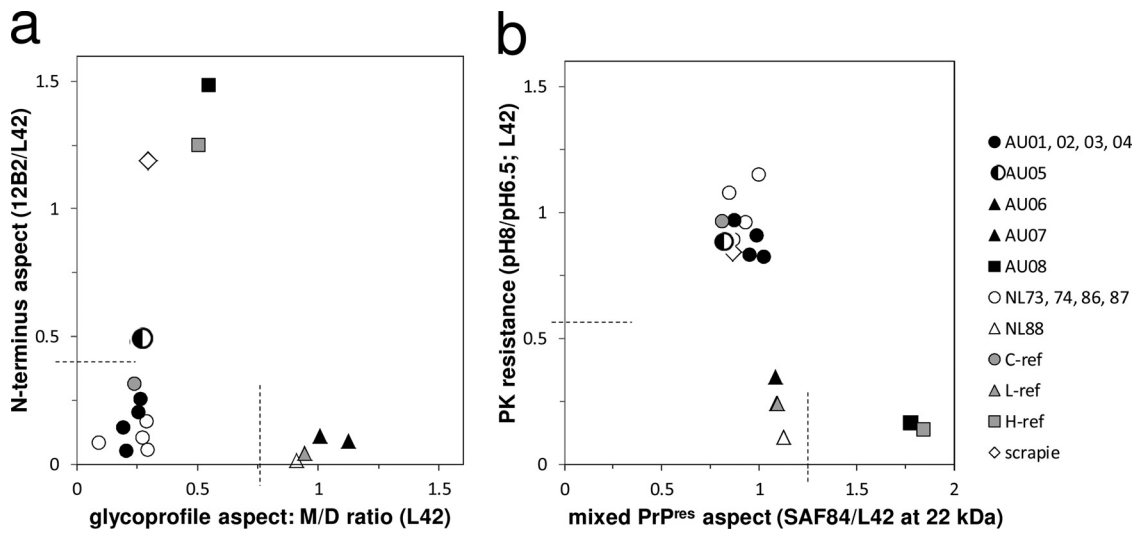

FIG. 1. Combined graphical presentations of four independent discriminatory PrP^{res} parameters for BSE isolate typing. (a) The vertical axis reflects the abundance of the N-terminal epitope of MAb 12B2 relative to the core epitope of MAb L42. On the horizontal axis, the ratio of monoglycosyl to diglycosyl forms (M/D) as probed with MAb L42 reflects a glycoprofile property of the triple-banded PrP^{res} pattern. (b) The vertical axis reflects the resistance to PK, measured as the L42 binding ratio between samples digested at pH 8 and pH 6.5. The horizontal axis with the SAF84/L42 ratio represents another glycoprofile aspect—values of >1.25 point to the existence of two populations of PrP^{res} triple bands in one sample (15). (a and b) By dividing the graphs each into four sectors, 1 to 4 (following the hands of the clock starting from the bottom left sector), the isolate types studied can be assigned to the following categories for C, L, and H BSE: A1B2, A4B1, and A2B4, respectively. The single classical scrapie case fits into category A2B2. The AU05 isolate is considered an exceptional C BSE case due to its N terminus aspect and, thus, having an intermediate behavior between the A1B2 and A2B2 categories.

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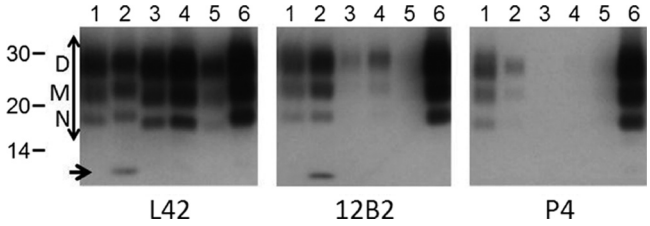

FIG. 2. Inspection of N-terminal epitope abundance with MAbs P4 and 12B2 relative to the PrP core L42 epitope. Lane 1, AU05; lane 2, reference H BSE; lane 3, C-type reference; lane 4, AU04; lane 5, AU02; lane 6, classical scrapie. Applied tissue equivalent (milligrams of tissue per lane) (TE) for lanes 1 to 4, 0.5 mg; for lane 5, 2.5 mg; for lane 6, 0.25 mg. The positions of the D, M, and N triple glycoprofile bands are indicated. The region for glycoprofile estimation is indicated with a double-headed arrow. The migration positions of three molecular mass standards are indicated in kDa. The M position coincides with the referred migration at 22 kDa (Fig. 1). A 7-kDa fragment is observed in H BSE samples (arrow).

16 categories can be constructed. The samples shown in our study fit into four of these: A1B2, A2B2, A4B1, and A2B4 for C BSE, AU05 (and scrapie), L BSE, and H BSE, respectively (Table 1). The most recent cases in Austria and the Netherlands were L type (AU06, AU07, NL88) and H type (AU08), while AU05 behaved as a C BSE case with a peculiar characteristic of an enhanced presence of the N-terminal 101WGQGG105 PrP sequence and unusually strong MAb P4 binding for bovine PrP. Unfortunately, no more AU05 tissue is available for strain typing, and the tissue was subject to strong autolysis, which might have influenced the outcome.

Considering the old ages of the BSE cases detected, it would be best to maintain BSE surveillance of cattle in the age cohort of at least 8 years and older.

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