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# Atypical status of bovine spongiform encephalopathy in Poland: a molecular typing study

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## Summary

The aim of this study was to analyze molecular features of protease-resistant prion protein ($PrP^{res}$) in Western blots of BSE cases diagnosed in Poland with respect to a possible atypical status. Confirmed cases were analyzed by Western blotting with several monoclonal antibodies directed at N-terminal and core epitopes of prion protein (PrP). Most cases showed the classical glycoprofile characterized by the dominance of the di- over the monoglycosylated $PrP^{res}$ band, yielding di-/mono- ratios well above 2 and by reactivity with antibodies having their epitopes in bovine PrP region 110–242 (C-type cases). Surprisingly, seven cases of BSE were atypical. Six were classified as L-type based on a slightly lower molecular mass ($M_r$) of the non-glycosylated band with respect to C-types and a conspicuously low di-/mono- ratio of glycosylated $PrP^{res}$ bands approaching unity. One case was classified as H-type because of a higher $M_r$ of $PrP^{res}$ bands on the blot when compared with C-type cases. A characteristic epitope of H-type $PrP^{res}$ occurred in the 101–110 region of PrP for which only antibody 12B2 had a sufficient affinity. The occurrence of atypical cases only in animals 9 years of age and older raises questions about the mechanisms of prion diseases and the origin of BSE.

## Introduction

A hallmark of prion diseases or transmissible spongiform encephalopthies (TSEs) is the deposition in the brain and some other tissues of a protease-resistant form of a host protein – prion protein (PrP). Depending on the diagnostic technique, this aberrant form is termed differently: $PrP^{d}$ for describing the unusual and symmetrical depositions in the brain, or $PrP^{res}$ as a molecular description for the protease-resistant form in immunochemical tests like enzyme-linked immunosorbent assay and Western blot [22, 26, 36].

Despite the fact that bovine spongiform encephalopathy (BSE) was described for the first time 20 years ago, there are still many issues related to the causative agent and the origin of the disease that are not fully elucidated [17, 18, 38, 39]. While at the time of discovery it was a new disease in cows, a similar disease in sheep, namely scrapie, has been known for hundreds of years [7, 24]. Therefore, scrapie is regarded as the prototype in a group of

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diseases called TSEs or prion diseases. Before the BSE-derived variant of Creutzfeldt-Jakob disease (vCJD) was recognized in humans, other forms of TSEs in man were already known, such as kuru, fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker syndrome (GSS) and sporadic, iatrogenic and familial forms of CJD [19].

In sheep, many strains have been described, pointing to quite a variety of biological features of the agent [11, 12]. On the other hand, although more than 180,000 cases of BSE have been diagnosed in the UK and more than 5000 in continental Europe, the agent from clinically affected animals seems to be very stable during an epidemic, based mostly on histology of brain lesions in cattle, but also on a limited set of bioassay studies in mice [9, 10, 16, 20, 23, 30, 38].

However, since 2003, unusual cases of BSE have been reported in France, Italy, Belgium, Japan, Germany, the United States and Switzerland [1, 6, 14, 17, 21, 35, 37, 42]. Those studies were mostly based on unusual molecular features of $PrP^{res}$ like a triple band pattern due to variations in glycosylation and migration positions because of variable, but often strain-type-dependent, cleavage by proteinase K. Two atypical forms of BSE have been defined: H-type (higher position on the gel with respect to classical BSE) and L-type (lower position when compared with classical BSE) [25]. Unusual features of those atypical cases were further confirmed in bioassay studies in both wild-type and in transgenic mice [3, 5, 14, 15]. Despite a species barrier, features of $PrP^{res}$ that were observed previously in cows were also maintained in mice. Similarities between $PrP^{res}$ from those atypical cases and the sporadic form of CJD (sCJD) in man and scrapie in small ruminants triggered further discussions about the origin of BSE [2, 6, 8, 17, 34]. Until now, no evident clinical features reminiscent of a neurological disease have been available for these atypical cases. Their clinical histories are not available because the detection was accomplished through active surveillance programs using biochemical tests on healthy slaughtered and fallen stock animals.

In Poland, the first case of BSE was detected in a healthy cow in May 2002 by active surveillance using postmortem testing, while until then no cases were found in small-scale passive surveillance in BSE-suspect animals conducted since 1996 [31, 33]. Until the end of 2006, 50 cases of BSE were diagnosed and confirmed in Poland. All of them were found in native-born cattle of black-and-white breed. An abberant glycoprofile of $PrP^{res}$ in an older animal was already reported in 2004 [32].

Therefore, the objective of this study was to perform a retrospective analysis of the molecular features of $PrP^{res}$ from all cases of BSE in search of atypical glycoprofiles and to classify them according to current knowledge and nomenclature. Positive homogenates from confirmed UK cases were used as reference material to get characteristics of classical BSE and to relate it to all available Polish cases. In this paper, we report a striking occurrence of atypical cases in a large fraction of BSE-infected animals 9 years of age and older. For recognizing H-type cases, N-terminus-specific antibody 12B2 appeared more suitable than antibody P4.

## Materials and methods

### *Brain stem tissue*

The majority of all positive cases were diagnosed initially during active surveillance using one of three rapid tests. Brain stem samples comprising the obex region were collected with a special spoon via *foramen magnum*. Confirmation was based on histopathology and immunohistochemistry. Only 2 out of 50 confirmed cases – ages 8 and 7 years – were initially diagnosed during passive surveillance, using formalin-fixed samples. Therefore, they were unsuitable for Western blot (WB) analysis. Additionally, for quality assessments, 16 positive homogenates from confirmed British cases of BSE, collected during passive surveillance, kindly provided by the Community Reference Laboratory, Weybridge, UK, were used in the study (UK samples) for comparison.

### *Western blot (WB)*

Material suitable for WB from 48 Polish cases was analyzed by means of a modified Prionics-Check Western (Prionics) rapid test [36]. In short, 10% homogenates of obex samples (w/v) were prepared in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in 10 mM PBS, pH 7.2) using an omni homogenizer (except for UK samples prepared in Prionics homogenisation buffer). Next, 1 ml of each homogenate was centrifuged at $1000 \times g$ for 5 min and supernatants were collected. Digestion with proteinase K (Sigma, 50 µg/

ml) was done at 37 °C for 60 min. After addition of digestion stop buffer (Prionics), samples were precipitated with an equal volume of 1-propanol (Sigma) as described [28]. Samples were centrifuged, supernatants were discarded and sediments were resuspended in 1× sample buffer (Prionics). The samples were denatured at 96 °C for 5 min before loading on 12% SDS-PAGE Bis-Tris gels (NuPAGE 12% Bis-Tris gel, Invitrogen). Electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis – SDS-PAGE) was run in MOPS buffer, initially at 150 V and after 15 min at 200 V, until the dye reached the bottom of the gel. Molecular weight standards (Sigma, Invitrogen) were loaded in each gel to facilitate determination of the molecular mass of each form of $PrP^{res}$. Wet protein transfer (Western blot) to a polyvinylidene difluoride membrane (Immobilon P, Millipore) was carried out at 150 V for 1 h at 4 °C. The membranes were incubated in blocking buffer (Prionics) for 30 min. Incubation with monoclonal antibodies (mAbs) in TBST (Tris-buffered saline supplemented with 0.05% Tween 20) was performed for 60 min at room temperature on a rocking platform. Primary antibodies used were: mAbs 6H4 and 94B4 as PrP-core-specific antibodies, and 9A2, 12B2, P4 as N-terminus-specific antibodies [25, 27, 28, 36]. N-terminus-specific rabbit serum RB1 was also used [29]. The membranes were washed in TBST three times for 2 min each. Then secondary antibody (goat anti-mouse conjugate coupled with alkaline phosphatase, Prionics) was added at 1/5000 dilution in TBST, and the membranes were incubated for 30 min at room temperature with the addition of streptavidin-alkaline phosphatase (Sigma) to reveal the molecular weight standards. The washings with TBST were done five times for 2 min each. Chemiluminescence detection was performed according to the Prionics procedure, and the membranes were developed on X-ray films after incubation with CDP-Star (Sigma) – an alkaline phosphatase substrate. For quantitation of $PrP^{res}$, films were digitally scanned and subsequently analyzed using ONE-Dscan software version 1.31 (Scanalytics). For each sample, the proportion of di-, mono-, and non-glycosylated $PrP^{res}$ fractions were calculated as percentages of total $PrP^{res}$, and mean values ± standard deviations (SD) were used for data analysis. Also, migration patterns in these scans were used for apparent mass determination of the respective forms of $PrP^{res}$, in each case by comparison with the migration distance of the molecular weight standard run on each gel. Each sample was tested in several repetitions on several gels.

In some cases, PK-digested homogenates were further deglycosylated with 50 U of PNGaseF (New England Biolabs) as described [25]. SDS-PAGE and Western blotting were carried out as described above, using 9A2 as primary antibody.

## Results

The data and graphs presented below for glycoform contents and molecular masses were obtained with antibody 6H4, except when explicitely indicated otherwise.

### *C-types*

Glycoprofiles and migration patterns of $PrP^{res}$ in analyses of UK samples provided a basis for classification as classical BSE (C-type) cases (Fig. 1). Mean proportions of the three forms of $PrP^{res}$ (di-, mono- and nonglycosylated moieties) were respectively 68, 24 and 8%. Apparent molecular mass ($M_r$) estimates of $PrP^{res}$ bands were as follows: 26.9, 21.2 and 17.2 kDa for the di-, mono-, and non-glycosylated bands, respectively. Analysis of $PrP^{res}$ from Polish cases of confirmed BSE showed similar proportions of $PrP^{res}$ moieties in

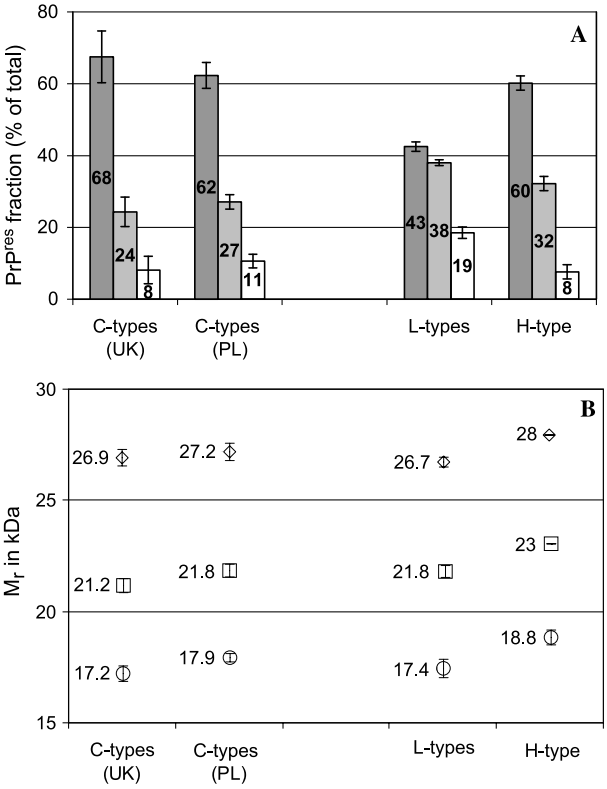


**Fig. 1.** Molecular $PrP^{res}$ profiles of distinct groups of BSE cases. (**A**) bar diagram for relative proportions of di- (*dark*), mono- (*grey*), and non-glycosylated (*white*) $PrP^{res}$ fraction expressed as percentage of all three fractions together. (**B**) graph representing apparent molecular mass of di- (*diamonds*), mono- (*squares*), and non-glycosylated (*circles*) $PrP^{res}$ bands. Values are given as averages ± SD

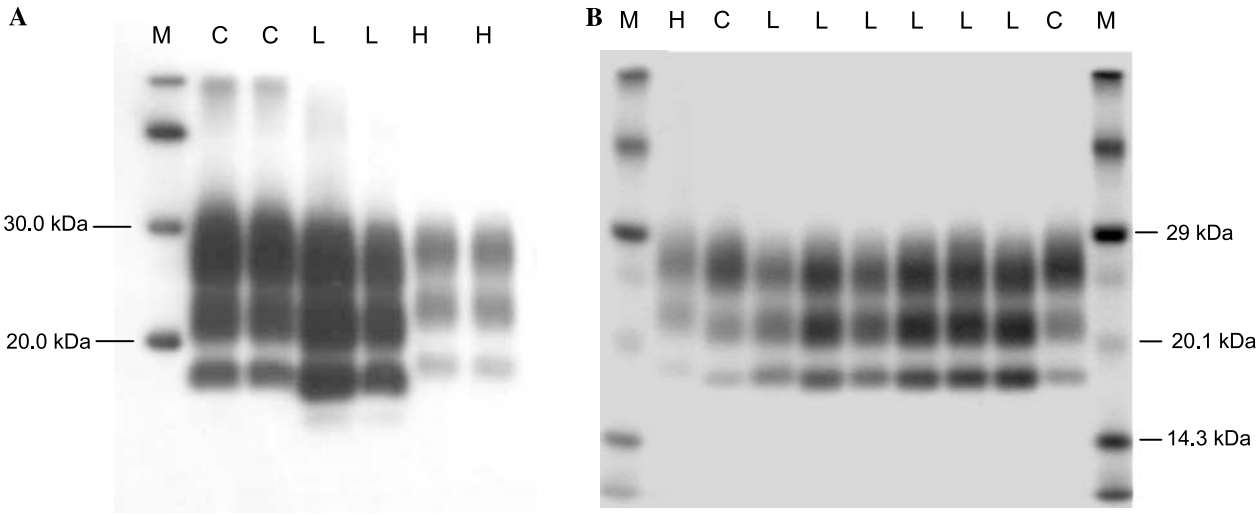


**Fig. 2.** Immunostaining of $PrP^{res}$ in different BSE-types. (**A**) $PrP^{res}$ in Polish BSE cases of different type detected with a core specific antibody 6H4. Migration characteristics of $PrP^{res}$ in Polish BSE-types loaded in duplicates (*M* molecular weight standard; *C* C-type case; *L* L-type case; *H* H-type case). (**B**) $PrP^{res}$ in Polish BSE-cases of different type detected with an N-terminus specific antibody 9A2. Every lane refers to one isolate except for C-type loaded twice (*M* molecular weight standard; *H* H-type case; *C* C-type case; *L* L-type case)

41 out of 48 homogenates tested (Figs. 1 and 2). These 41 cases, like the British cases, contained a high relative amount of the diglycosylated form (62%) and much lower fractions of mono- (27%) and nonglycosylated bands (11%). $M_r$ of di-, mono- and non-glycosylated $PrP^{res}$ moieties were on average 27.2, 21.8 and 17.9 kDa, respectively. The values differ from those obtained above for the British cases, but they are still within the range of variations measured for individual samples between different experiments (interassay variation) (coefficient of variation equal to 2.4 kDa for UK samples and 1.3 kDa for Polish samples).

These C-type isolates reacted with core antibodies and with N-terminus-specific mAb 9A2 (epitope mapped to 110–112 of bovine PrP). However, there was hardly any reactivity with mAbs 12B2 and P4 (epitopes mapped respectively to amino acids 101–105 and 101–107 of bovine PrP) located further to the N-terminus of PrP (Fig. 3). The age of these C-type cases ranged from 2 to 12 years.

The 7 remaining cases will be discussed in the next paragraphs.

*L-types*

Different glycoprofiles were observed in 6 cases, all from older cattle aged 9 years and more. Mean proportions of the three forms of $PrP^{res}$ (di-, mono- and non-glycosylated moieties) were 43, 38, and 19%, respectively (Fig. 1A). Thus, compared to the C-type cases, the relative amount of diglycosylated $PrP^{res}$ in L-types was lower, resulting in a higher mono- and non-glycosylated fraction. The $M_r$ values of the di-, mono- and nonglycosylated moieties were 26.7, 21.8 and 17.4 kDa, respectively (Fig. 1B). This slightly faster migration of the nonglycosylated band was not very obvious upon visual inspection, but it was consistently present when compared in the same run with C-type cases. Also, a distinction could be made with mAb 6H4, but not with 9A2 (Fig. 2). However, when compared to the C-type cases, the low amount of the diglycosylated form and higher amount of the monoglycosylated form was a constant feature. The ratio between the di- and monoglycosylated fractions was strikingly invariant, showing only 5–10% fluctuation in the diglycosylated/monoglycosylated ratio (D/M ratio), which was lower than that found in the group of C-type cases (Table 1).

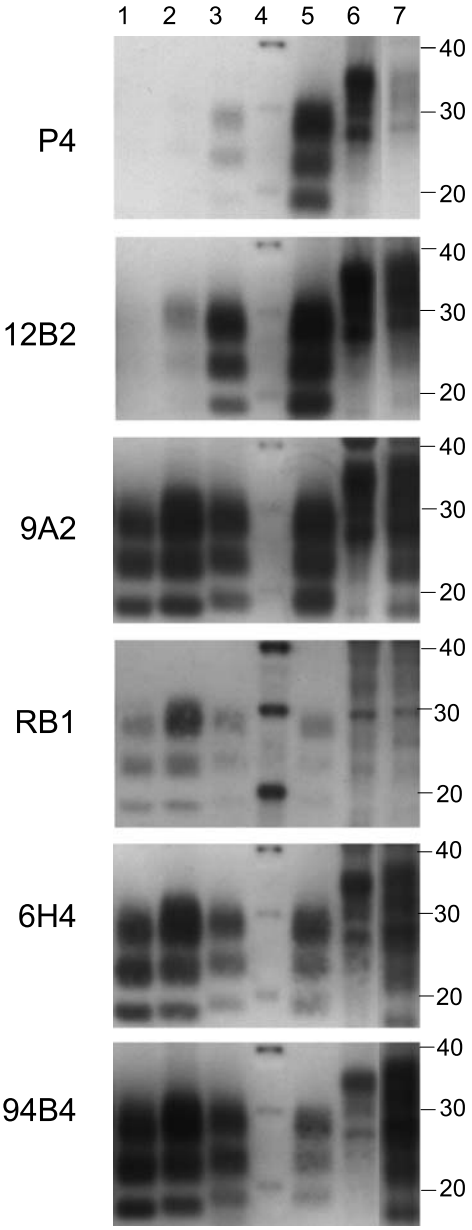


**Fig. 3.** Antibody 12B2 as preferential tool for detection of H-type BSE: $PrP^{res}$ N terminus (P4, 12B2, RB1 and 9A2) and core specific antibodies (6H4 and 94B4) applied on bovine and ovine brain samples. *1–3* and *7* Bovine brain stem tissue. *5–6* ovine brain stem tissues. *1* L-type PL28, *2* C-type PL1, *3* H-type PL45, *4* molecular weight standard (XP, Invitrogen, values in kDa), *5* scrapie, *6* undigested ovine, *7* undigested bovine. Tissue equivalents: 0.5 mg/lane. The epitopes of the antibodies 12B2, P4, 9A2 and RB1 are very closely located in bovine PrP, namely at 101–105, 101–107, 110–112, and 110–113, respectively

**Table 1.** Ratios of diglycosylated to monoglycosylated $PrP^{res}$ as indicator for L-type cases

| BSE-group | D/M ratio ± SD (range) |
|---|---|
| C-types (UK) | 2.5 ± 0.5 (2.0–3.6) |
| C-types (PL) | 2.2 ± 0.2 (1.9–2.6) |
| L-types | 1.1 ± 0.04 (1.1–1.2) |
| H-type | 1.9 |

D/M ratio, ratio of percentage diglycosylated to monoglycosylated $PrP^{res}$. Average values were taken for C-types UK, C-types PL, L-types and H-type from 16, 41, 6, and 1 cases, respectively. Western blots were immunostained with mAb 6H4, and several runs of each sample were tested. The variation for the single H-type case in repeated analyses was 1.9 ± 0.1 (1.8–2.1), 6 runs.

### *H-type*

An additional type of $PrP^{res}$ was found in a 10-year-old BSE cow. A striking feature in this case was a higher $M_r$ of all three bands, resulting in clearly higher positions on the blot when using antibodies with epitopes located in the 110–242 region of PrP. The $M_r$ values for di-, mono- and nonglycosylated forms were 28.0, 23.0 and 18.8 kDa, respectively (Fig. 1B). The glycoprofile of this case resembled the classical type with 60% diglycosylated, 32% monoglycosylated, and 8% nonglycosylated form (Fig. 1A, Table 1). Due to higher migration of PrP glycoforms in SDS-PAGE, this type of BSE was termed H-type (Fig. 2).

### *Comparison of N-terminus-specific antibodies for BSE-type discrimination*

From the above it was evident that, in H-type BSE cases, proteinase K cleaved the N-terminal domain of PrP only till residue 101 or even closer to the N-terminus of the bovine PrP sequence, instead of between residue 101 and 110 as in C-type or L-type BSE. When several available N-terminus-specific antibodies were compared for their affinity to the different types of BSE and scrapie from sheep, it became clear that only mAb 12B2 (binds to PrP region 101–105) exhibited similar affinity to $PrP^{res}$ of H-type BSE cases as core-specific antibodies and N-terminus-specific antibodies 9A2 and RB1, which bind to bovine PrP region 110–113 (Fig. 3). However, although P4 and 12B2 have sim-

ilar epitope specificities [28], P4, in contrast to 12B2, bound only poorly $PrP^{res}$ and intact PrP from cattle (see Fig. 3, lanes 3 and 7), while both antibodies bound well to $PrP^{res}$ and intact PrP from sheep (see Fig. 3, lanes 5 and 6).

### *Deglycosylation*

Deglycosylation results obtained by digestion with PNGase F showed that both the top and middle bands of the triple band components of $PrP^{res}$ were truly glycosylated in all BSE cases, since they were absent after deglycosylation, and only a single nonglycosylated form was observed (Fig. 4). A striking phenomenon was that bands resulting from deglycosylation with PNGase F from all three types of BSE were broader and thus exhibited an average higher apparent mass (higher position on the blot) than the nonglycosylated undigested counterparts (compare lanes − and + in Fig. 4). This suggests a slightly different physical behaviour between nonglycosylated $PrP^{res}$ after PK digestion only and deglycosylated $PrP^{res}$ after additional enzymatic deglycosylation with PNGase F.

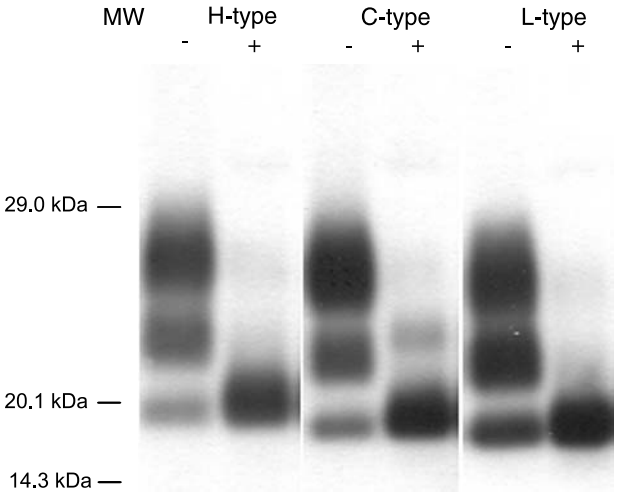


**Fig. 4.** Deglycosylation of three types of BSE subjected to digestion with PNGase F. Antibody used for detection: 6H4 (0.2 μg/ml). *Symbols*: − nondigested samples, + digested samples, *MW* molecular weight standards. Applied amounts: 0.3 mg of tissue equivalent per lane

### *Details about atypical cases*

All seven atypical cases were initially detected during active surveillance by means of one of two rapid tests in use at the time of screening in Poland (Table 2). The cases were all older animals, 9–13 years of age (mean age of 11 years), while classical BSE was found in animals 2–12 years old (mean age of 7 years). None of these cases was reported to have clinical signs, although this could not be further investigated. All cases were thus detected by rapid tests, indicating the suitability of these tests for detection, despite the above-mentioned variations between the BSE types with respect to their dependence on N-terminal epitopes and the migration of $PrP^{res}$. Every case except case no. 2 had high

**Table 2.** Detailed information on cases of atypical BSE diagnosed in Poland

| Case no. | BSE type | Year detected | Age (years) at detection | Health[a] status | Rapid test results (cut off)[b] | | Confirmatory test results[c] |
|---|---|---|---|---|---|---|---|
| 2 | L | 2002 | 12.6 | h.s. | 5.56 (5.5) | E | +/+/+ |
| 15 | L | 2004 | 9.4 | h.s. | 3.46 (0.22) | B | +/+/+ |
| 17 | L | 2004 | 12.6 | e.s. | 3.38 (0.22) | B | NT/+/+ |
| 28 | L | 2005 | 11.2 | h.s. | 3.27 (0.23) | B | +/±/+ |
| 31 | L | 2005 | 13.2 | f.s. | 3.34 (0.22) | B | NT/+/+ |
| 43 | L | 2006 | 12.1 | f.s. | 0.821 (0.22) | B | NT/+/+ |
| 45 | H | 2006 | 10.2 | h.s. | 2.83 (0.22) | B | NT/+/+ |

[a] Health status at detection: *h.s.* healthy slaughter, *e.s.* emergency slaughter, *f.s.* fallen stock.
[b] Rapid test result: *E* Enfer TSE ELISA, *B* Biorad TeSeE ELISA.
[c] Confirmatory test results are from three tests: histopathology by HE staining and vacuoles, IHC and Western blot. A modified Prionics Check Western procedure (digestion at 37 °C for 60 min) was used in the Western blot. *Symbols*: + positive, ± weakly positive, *NT* not tested.

readings in the rapid test, although case no. 2 was also strongly $PrP^{res}$ positive in subsequent analysis by Western blot. All of the atypical cases were confirmed using histopathology, immunohistochemistry and/or modified Western blot, and each case was clearly positive.

## Discussion

The molecular data presented in this study clearly show the existence of atypical BSE in older cattle in Poland. Some special diagnostic features for confirmatory discrimination from C-type cases are presented, such as for L-type cases, a rather consistent 1/1 ratio between the di- and monoglycosylated $PrP^{res}$ fractions (D/M ratio) and for the H-type case, the strong binding of the $PrP^{res}$ triplet to an antibody, 12B2. The first atypical BSE case in Poland was an L-type detected in 2002 and diagnosed as atypical in 2004 [31, 32]. Altogether in the present study, until the end of 2006, seven BSE cases (comprising 14% of all positives) were classified as atypicals based on their glycoprofile, migration pattern and antibody binding of $PrP^{res}$. All of those cases were diagnosed during active surveillance, but analysis of $PrP^{d}$ distribution patterns in different regions of the central nervous system was impossible since only brain stem samples could be obtained. The TSE strain status of some of these cases is under investigation in bioassays.

The recognition of an atypical BSE status requires a clear definition of classical forms of the disease. Even though critical data on BSE-type classification by molecular $PrP^{res}$ analyses have been described, two features are used here that would better assist a robust typing than indicated in those studies [25]. Firstly, as described, $PrP^{res}$ from classical BSE was considered to be characterized by a predominance of the diglycosyl form, but this is also the case in H-type cases. However for H-type cases, this depends on the antibody used, and therefore the $PrP^{res}$ glycoprofile is a debatable indicator for discrimination between H-type and C-type BSE. However, L-type cases can be recognized based on the criterium of the glycoprofile due to the high relative fraction of non- and mono-glycosylated $PrP^{res}$. While variations were observed in terms of percentage values of $PrP^{res}$ glycoforms, in cases analyzed, the ratio between diglycosylated and monoglycosylated $PrP^{res}$ (D/M) proved to be a very reliable indicator for recognition of L-types (Table 1). The D/M ratios for all British and Polish C-type cases analyzed were 1.9 and higher. The H-type case also exhibited a C-type-like D/M ratio (values between 1.8 and 2.1). Remarkably, the six L-type cases had consistently low D/M ratios, between 1.1 and 1.2, with little variation between and within cases. Secondly, as expected, the slower migration of $PrP^{res}$ bands was the most conspicuous tool for detection of the H-type case, as also observed previously [6, 14, 25]. This migration feature was very clear with both N-terminus-specific antibodies (9A2, RB1) and core-specific antibodies like 6H4 and 94B4. While migration differences between runs often yield different $M_r$ values, antibody reactivities remain independent of such fluctuations. It was shown here however, that for immunochemical discrimination between H-type and C-type, antibody 12B2 was the most powerful, since only with the H-type case did the antibody yield approximately equal signals in density compared to antibodies 6H4, 9A2, and 94B4. Antibody P4, with a somewhat larger epitope requirement than 12B2, was subject to species-dependent sequence variations which made it a less suitable candidate for application to bovine PrP.

Migration differences between C-type $PrP^{res}$ and atypical cases were in agreement with published observations. When using core-specific antibody 6H4, the difference between H-type and C-type was estimated to be 0.9–1.2 kDa larger $PrP^{res}$ molecules for H-type than C-type. In contrast, $PrP^{res}$ molecules of L-type cases within single experiments were consistently up to 0.5 kDa smaller than those of C-type cases. This faster migration, especially of the nonglycosylated band, has been described as one of the key features of BASE or L-type cases of BSE [17], but our experience shows that it may be difficult to notice this difference under standard WB conditions. A similar conclusion was drawn from a German L-type case, where the only clear difference from classical BSE was the predominance of the diglycosylated form of $PrP^{res}$ [14]. With N-terminus-specific antibody 9A2, the

migration pattern of the L-type was even indistinguishable from C-type cases, which can be explained by the fact that cleavage of $PrP^{res}$ in L-type cases by proteinase K occurs directly at and in the epitope of this antibody (residue 110–112) so that only the larger size population can be visualised, while core-specific antibodies bind to both shorter and larger-size $PrP^{res}$ populations.

A common feature of all atypical cases was the higher age of affected individuals (9–13 years) compared to classical BSE found in various age groups (2–12 years). Opposite to our findings, first in Japan and then in Belgium, atypical cases of BSE were found in younger cattle [21, 42]. The Japanese case was found in a 23-month-old steer, while the Belgian cow was 64 months old. Further studies performed in other countries where atypical BSE was found (France, Italy, Germany, Sweden, The Netherlands, USA) showed that only older cattle (8 years and older) were affected [25, 35]. While most of these cases could be properly identified, comparing cases described in Japan and Belgium with atypical BSE found in Europe, one can presume that those were L-types due to a low content of the diglycosylated form and faster migration of the nonglycosylated moiety of $PrP^{res}$. An additional feature found in the Japanese case, namely less resistance to PK digestion in comparison to classical BSE, resembled atypical scrapie [4, 13]. However, comparison between studies for glycoprofiles can only properly be performed with the same reference samples for comparisons and using similar antibody conditions. The identity of the BSE type cannot be confirmed, either for the Belgian or the Japanese case since histopahological and immunohistochemical data are lacking, while all Polish atypical cases were confirmed by at least two confirmatory methods. Further collaborative studies within the NeuroPrion network of excellence in Europe on atypical cases of BSE has enabled the distinction of L-type BSE with molecular features similar to BASE, and H-type BSE in several countries with a practical approach for the identification of such cases [25]. Our six Polish cases described had similar molecular characteristics of $PrP^{res}$ to Italian BASE and all of them could be assigned to L-type BSE.

Atypical cases in Poland were found almost equally in both healthy (57%) and risk-group (43%) cattle, but the majority of classical BSE in Poland belonged to the healthy slaughtered group (79%), which is probably directly correlated with testing volume (majority of tested samples coming from healthy slaughtered animals). Therefore, despite testing mostly healthy slaughtered animals, atypical BSE seems to be dominant in risk-group cattle. But this hypothesis can only be properly tested when more atypical cases have been found.

The percentage of samples tested in Poland from animals 8 years and older comprised 1% in 2002, 23% in 2003, 52% in 2004, 54% in 2005, and 53% in 2006. Therefore, it can be speculated that the relatively high number of L-type BSE in Poland may be correlated with a high percentage of older cattle tested within the active surveillance scheme. Despite the constantly growing number of samples tested in 2004–2006 (increase from 481,116 samples tested in 2004 to 625,438 samples tested in 2006), only 2 atypical cases were found per year. When geographical distribution was taken into account, atypical cases were predominant in eastern Poland (5 out of 7 cases comprising 71%), while the remaining 2 cases were located in the central part. Classical BSE was distributed more evenly, with 44% in the central part, 30% in the eastern part, and 26% in the western part of Poland. Polish atypical cases require more detailed analysis in relation to geographical distribution and other factors before any general statement related to epidemiology or risk can be made.

Clarification of whether these atypical cases represent genuine strains of BSE would be accomplished by transmission studies in mice. Such studies have already been performed in France, Germany and Italy [3, 5, 14, 15]. For H-type cases in France, successful transmission was achieved in both wild-type, and transgenic mice expressing bovine and ovine $PrP^{C}$. In Germany, successful transmission of both an L-type and an H-type case to transgenic mice overexpressing bovine $PrP^{C}$ has been described. $PrP^{res}$ from those mice was identical to the inoculum used in the study, proving the existence of distinct strains of BSE. All atypical features of those isolates were maintained in the

inoculated mice, indicating the existence of several prion strains in cattle, or alternatively a possible evolution to a single BSE strain, as suggested from data obtained by Capobianco et al. with wild-type inbred mice [15]. This second hypothesis could fit with data from the United Kingdom, where over 180,000 cases of BSE were diagnosed by passive surveillance. British and European experience based on tissue analysis from clinically affected animals showed consistent characteristics of BSE agent not only on histological sections from cattle brains but also when inoculating mice, pointing to the existence of one uniform strain of BSE. Therefore, it is possible that a sporadic form of BSE present in the cattle population at a very low rate in the past could have spread to naive animals via contaminated meat-and-bone meals. Spontaneous BSE, if it occurs, must be a very rare phenomenon. However, data for Poland, where 14% of all cases comprised an atypical form of BSE, seems to be in contradiction to this hypothesis. But when the average age of all positive cases in Poland is taken into account, BSE is generally found in older animals (mean age of 7.7). Analysis of the age structure of cattle in Poland in the period of 2002–2006 shows that 56–60% of all animals were 7 years old and above. A much larger number of cattle should be tested to get better insight into the real prevalence of atypical BSE. However, current tendencies based on economic analysis point to a decrease in the number of tests performed rather than expanding this scheme any further. It would be sensible to maintain a certain level of testing focused on the older age group to distinguish between a stable, thus sporadic-based, situation of BSE, or alternatively a fade-out, thus epidemic-based, situation. Exploring the subject of spontaneous BSE in the cattle population may be ceased for economic reasons, and it may never be known while this answer is in our reach thanks to great financial efforts in recent years.

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