

## **PRION PROTEIN GLYCOFORMS FROM BSE CASES IN POLAND**

MIROSLAW P. POLAK, WOJCIECH ROZEK, JERZY ROLA AND JAN F. ZMUDZIŃSKI

Department of Virology,  
National Veterinary Research Institute,  
24-100 Pulawy, Poland  
e-mail: ppolak@piwet.pulawy.pl

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### **Abstract**

The article describes the analysis of proteinase K resistant prion protein from Polish cases of bovine spongiform encephalopathy (BSE). Brainstem samples from 12 out of 14 cases of BSE diagnosed so far in Poland have been used in the study. Glycotyping showed comparable molecular sizes of three glycoforms in all cases and typical for BSE predominance of diglycosylated form of PrP<sup>res</sup> in 11 samples. In one case equivalent relative amounts of both di- and monoglycosylated forms of prion protein were detected. The results were compared with both human and other animal TSEs glycotyping studies of PrP<sup>res</sup> and the reference was made to the possibility of existence of BSE strains on the basis of characteristic glycoforms of PrP<sup>res</sup>.

**Key words:** bovine spongiform encephalopathy, glycoforms, PrP<sup>res</sup> typing, prion strains.

Bovine spongiform encephalopathy (BSE) is a transmissible spongiform encephalopathy (TSE) of cattle with characteristic lack of immunological response, extended incubation period and 100% fatal outcome. Scrapie in sheep and Creutzfeldt-Jakob disease in humans are other diseases belonging to the same group of neurodegenerative disorders, also known as prion diseases (13).

The hallmark of BSE pathogenesis is the post-translational conversion of host-encoded prion protein (PrP<sup>C</sup>) to its pathological isomer defined as PrP<sup>Sc</sup> or PrP<sup>res</sup> according to its partial resistance to proteolysis. The prion protein has two N glycosylation sites and is present in the cell in three glycoforms (di-, mono-, and unglycosylated form) (8). In human TSEs it has been shown that differences in PrP<sup>res</sup> glycosylation may be responsible for strain-specific features of prion protein defining specific biological and biochemical properties (5, 7, 12).

Histopathology, immunohistochemistry and western-blot can be used to characterize localisation and magnitude of spongiform changes, length of incubation period in experimental rodent models, and PrP<sup>res</sup> glycosylation pattern (relative amount of di-, mono-, and

unglycosylated forms of PrP<sup>res</sup> as well as their sizes defined by migration distance in SDS-PAGE gel). Until recently, all available tests used to characterise BSE causative agent showed the same histopathological lesion profile, the same incubation period and similar glycotypes which indicated the existence of a single strain of BSE (1, 9, 11). Western blot analysis of PrP<sup>res</sup> glycoforms from BSE cases showed that the diglycosylated fragment was the most abundant and the size of the unglycosylated form defined by its electrophoretic mobility was unchanged. The opposite was for scrapie and CJD where, at least several different strains were described by means of the same criteria (2-5, 7, 10, 12, 15).

In March 2004, a group of Italian researchers described a molecular variant of bovine spongiform encephalopathy (6). Opposite to typical BSE, monoglycosylated form was predominant and all forms of PrP<sup>res</sup> showed faster electrophoretic mobility. These features were similar to PrP<sup>res</sup> of sporadic Creutzfeldt-Jakob disease. This discovery indicated the possibility of existence of a new strain of BSE potentially pathogenic for humans (probable appearance of a new disease in relation to BSE, like variant CJD).

Until the end of April 2004, 14 cases of BSE have been detected in Poland, all in native born cattle. Approximately 1 million samples have been tested so far within active monitoring framework using rapid tests. While 11 from 14 BSE cases discovered in Poland came from healthy animals, slaughtered for human consumption, only three cases detected so far were diagnosed in risk animals. Two of these cases were found in clinical suspects and one was diagnosed in dead farm animal.

The objective of the study was to compare Polish cases of BSE at molecular level in terms of electrophoretic rate of migration and glycoform content of prion protein in respect to the possible existence of molecular variants of pathological form of PrP resistant to proteinase K digestion. Two cases (the 4<sup>th</sup> and 5<sup>th</sup> case) were not analysed (brainstem samples were formalin-fixed).

## Material and Methods

Brainstem samples comprising obex region were collected with a special spoon via *foramen magnum*. Twelve brainstem samples were analysed by means of a hybrid Prionics-Check western blot, which is a modified version of Prionics-Check (Prionics) rapid test (14). In short, 1 ml of a 10% (w/vol) homogenates of obex region samples were centrifuged at 1 120xg for 5 min (Eppendorf microcentrifuge) followed by proteinase K (Prionics-Check) digestion at 37°C for 60 min. After addition of Digestion Stop buffer and Sample Buffer (Prionics-Check), the samples were boiled at 100°C for 10 min and centrifuged in a microcentrifuge (10 000xg for 5 min) before loading on 12% SDS-PAGE gels (NuPage gels, Invitrogen). Each sample was loaded in five wells of the gel. Electrophoresis at 100 V was carried out until the dye reached the bottom of the gel. Prestained molecular weight marker (Fermentas, Lithuania) was loaded in the first and the last well of each gel to facilitate determination of the molecular mass of each form of PrP<sup>res</sup>. Electrotransfer was done with polyvinylidene difluoride membrane (Immobilon P, Millipore) at 150 V for 1 h at 4°C. The membrane was blocked in Blocking Buffer (Prionics-Check) for 60 min. Incubation with primary antibody 6H4 (Prionics-Check) at 1/5 000 dilution in Blocking Buffer was done overnight at 4°C. The membrane was then washed in Tris-buffered saline supplemented with 0.05% Tween 20 (TBST) four times for 7 min each. Then secondary antibody (goat anti-mouse conjugate coupled with alkaline phosphatase – Prionics-Check) was added at 1/5 000 dilution in TBST and the membrane was incubated for 60 min at room temperature. The washings with TBST were done four times for 7 min each. Chemiluminescent detection was done according to Prionics procedure and the membrane was developed on x-ray film after incubation with an alkaline phosphatase substrate, CDP-Star (Sigma). The positions of molecular weight markers were marked on the film.

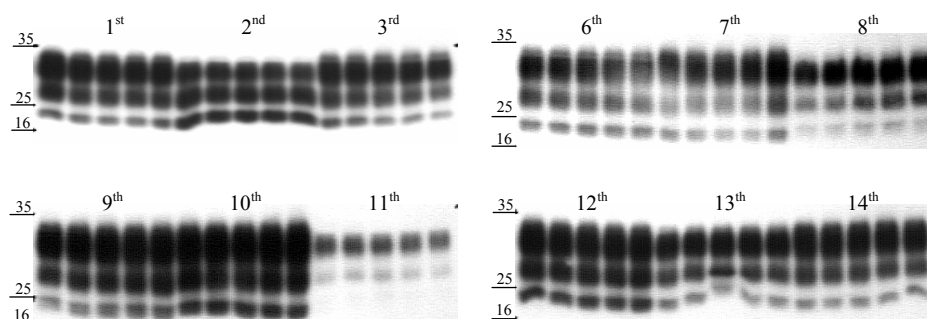
Gene Profiler software ver. 4.03 (Scanalytics) was used for analysis of digitally scanned membranes. Percentages of di-, mono-, and unglycosylated forms of PrP<sup>res</sup> were calculated for each sample (relative integrated density of a band, relative to the sum of all the bands in a lane) and mean values were used for statistical analysis (Student's t-test). Also migration patterns of respective forms of PrP<sup>res</sup> for each case were tested in the same way based on comparisons with the migration of molecular weight standard ran in duplicate on every gel. Each sample was tested in five repetitions (3 samples per gel).

## Results

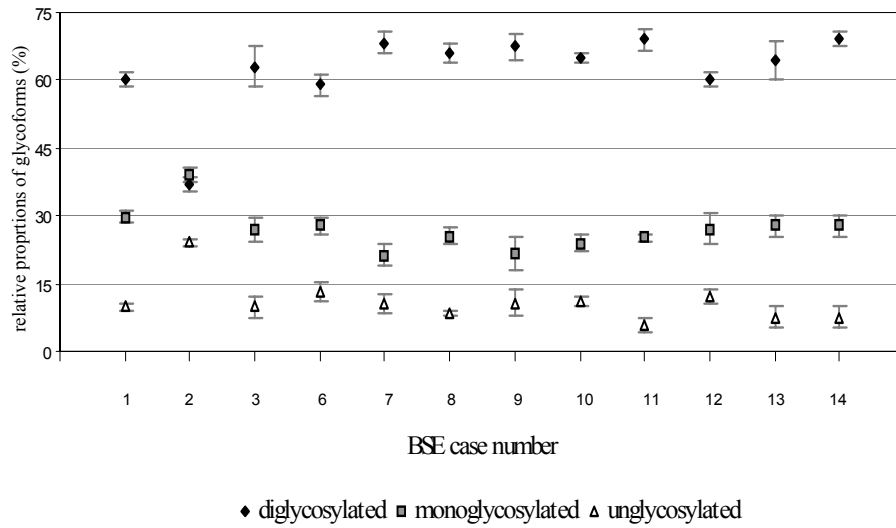
The relative amounts of specific forms of PrP<sup>res</sup> were similar for 11 cases (Figs 1, 2).

The diglycosylated band was the most abundant opposite to the amount of unglycosylated form. The second case of BSE showed different pattern of relative amounts of PrP<sup>res</sup> with monoglycosylated form being the most abundant (Fig. 2). However, PrP<sup>res</sup> migration pattern for this case was comparable with other cases (Fig. 3). On the basis of rate of migration of unglycosylated band, it may be suggested that PrP<sup>res</sup> for sample no. 7 was the smallest while the opposite was for sample no. 11. These differences were statistically significant ( $P < 0.05$ ) for samples no. 1, 2, 8, 9, 11, 12 when compared with case no. 7 and no. 1, 2, 3, 6, 7, 8, 12, 13, 14 compared with case no. 11.

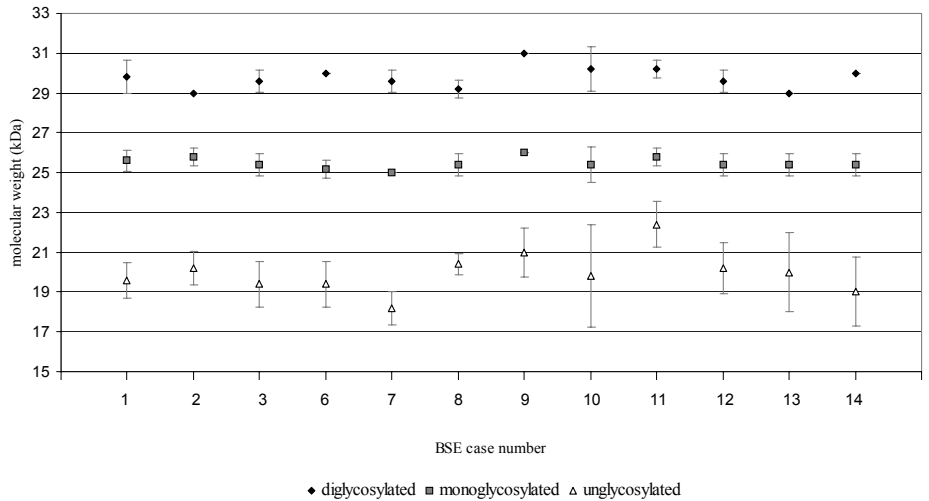
When percent ratio of diglycosylated to monoglycosylated form was plotted on a scattergraph, the second case was clearly separated from other cases, which clustered together in a different position on the graph (Fig. 4). This glycoform ratio for the second case was 37:39, while the ratio for all the remaining cases was from 59:28 to 69:24 ( $P < 0.05$ ).



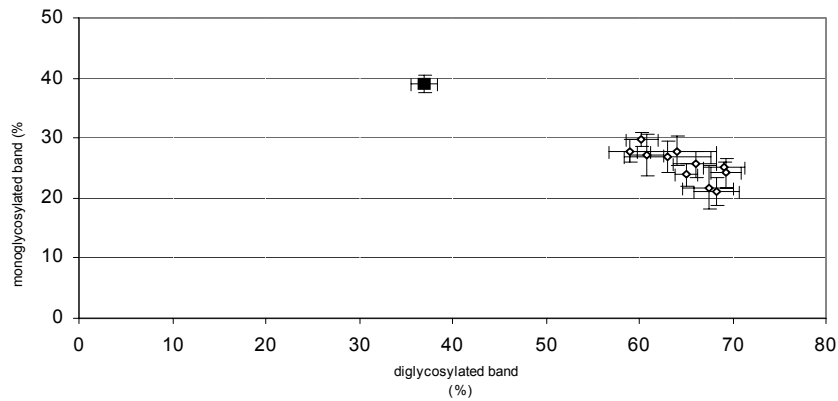
**Fig. 1.** Western blot of 12 Polish cases of BSE. Each sample was loaded into five wells of the gel, three samples on each gel. Numbers refer to the order of the appearance of the cases. Molecular size markers are shown on the left side of each gel.



**Fig. 2.** Scattergraph of relative proportions of PrP<sup>res</sup> glycoforms (means ± standard deviations) detected in Polish cattle with BSE.



**Fig. 3.** Migration distances for PrP<sup>res</sup> glycoforms based on their molecular weights (means ± standard deviations).



**Fig. 4.** Scattergraph of the glycoform ratio for the diglycosylated form versus the monoglycosylated form (means ± standard deviations). Glycoform ratio for the second case is marked with the black square.

## Discussion

Molecular typing of PrP<sup>res</sup> based on glycosylation profiles was used to characterize many scrapie cases (2-4, 9, 10, 14, 15). Similar PrP glycotyping was used in human prion diseases (5, 7, 12). Different electrophoretic mobilities of unglycosylated form and various ratios of PrP<sup>res</sup> glycoforms enabled the distinction of 2 to 4 types of pathological forms of prion protein which could be matched with different clinical forms of CJD. In the BSE cases analysed using histopathological, immunohistochemical and immunoblotting techniques no differences were observed despite different geographical origin of the analysed samples (1, 9, 11). Suitability of glycotyping of PrP<sup>res</sup> from scrapie and CJD cases for distinction of different strains prompted many researchers to look for such characteristics in cattle. Most of cases analysed, both from cattle and mice experimentally infected with BSE gave similar results and they were comparable with sheep scrapie (3). Sometimes only minor differences in migration pattern of unglycosylated form were observed between sheep and cattle TSEs (10).

In March 2004 Casalone *et al.* (6) described a second type of TSE in cattle. Opposite to typical BSE they found amyloid plaques and different pattern of regional distribution of PrP<sup>Sc</sup>. Western blot analysis showed that monoglycosylated form of PrP<sup>res</sup> was the most abundant and the molecular sizes of all three forms were smaller when compared with typical cases of BSE. Additionally, affected animals were the oldest in the group under study, which was not the case for Polish BSE affected cow (4 years old, while the oldest cases were 10 and 12 years old). Second Polish case of BSE showed almost identical glycoform ratio (37:39:24) when compared with Italian second type of BSE (37:38:25), however, migration distance was typical for other BSE cases.

Despite case no. 2, the results of the glycoform ratio analysis reported in our studies are in agreement with other findings confirming typical for BSE high diglycosylated to monoglycosylated ratio (7, 9, 10). Also the relative amounts of all three forms of PrP<sup>res</sup> in Polish cases were very similar to other studies (15). Sweeney *et al.* (15) showed that the amount of di-, mono-, and unglycosylated form, was 60-70%, 20-30% and 5-12%, respectively. In our studies these numbers were: 59-69%, 21-30%, and 6-13% for all cases except case no. 2. The second case had similar levels of di- and monoglycosylated forms (37 and 39%, respectively) while the unglycosylated band comprised 24%. Such a glycoform ratio was described for scrapie isolates (2) with the highest amount of monoglycosylated form rather than diglycosylated. Therefore it can be speculated that Polish BSE case no. 2 could be derived from sheep scrapie. But such assumption based only on glycoform pattern studies is not enough justified to draw such a conclusion. Moreover, other studies showed no differences between scrapie and BSE isolates when respective glycoforms were compared (3, 4, 7).

The results of this study show that most of Polish BSE cases have typical glycoform pattern, present also in BSE cases from other countries. The only exception to this rule is the case no. 2 with distinct features which might suggest ovine origin. However, accurate identification of such a linkage requires further analysis.

## References

1. Badiola J.J., Monleon E., Monzon M., Acin C., Lujan L., Fernandez D., Simmons M., Vargas A.: Description of the first cases of BSE in Spain. *Vet Rec* 2002, **151**, 509-510.
2. Baron T.G., Biacabe A.-G.: Molecular analysis of the abnormal prion protein during coinfection of mice by bovine spongiform encephalopathy and a scrapie agent. *J Virol* 2001, **75**, 107-114.
3. Baron T.G.M., Madec J.-Y., Calavas D.: Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. *J Clin Microbiol* 1999, **37**, 3701-3704.
4. Baron T.G.M., Madec J.-Y., Calavas D., Richard Y., Barillet F.: Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. *Neurosci Lett* 2000, **284**, 175-178.
5. Cardone F., Liu Q.G., Petraroli R., Ladogana A., D'Allesandro M., Arpino C., Di Bari M., Macchi G., Pocchiari M.: Prion protein glycoform analysis in familial and sporadic Creutzfeldt-Jakob disease patients. *Brain Res Bull* 1999, **49**, 429-433.
6. Casalone C., Zanusso G., Acutis P., Ferrari S., Capucci L., Tagliavini F., Monaco S., Caramelli M.: Identification of a second bovine amyloidotic spongiform encephalopathy: Molecular similarities with sporadic Creutzfeldt-Jakob disease. *Proc Natl Acad Sci USA* 2004, **101**, 3065-3070.
7. Collinge J., Sidle K.C.L., Meads J., Ironside J., Hill A.F.: Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. *Nature* 1996, **383**, 685-690.
8. Ermonval M., Mouillet-Richard S., Codogno P., Kellermann O., Botti J.: Evolving views in prion glycosylation: functional and pathological implications. *Biochimie* 2003, **85**, 33-45.
9. Kuczius T., Haist I., Groschup M.H.: Molecular analysis of bovine spongiform encephalopathy and scrapie strain variation. *J Infect Dis* 1998, **178**, 693-699.
10. Nonno R.M., Eseposito E., Vaccari G., Conte M., Marcon S., Di Bari M., Ligios C., Di Guardo G., Agrimi U.: 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* 2003 **41**, 4127-4133.
11. Orge L., Simas J.P., Fernandes A.C., Ramos M., Galo A.: Similarity of the lesion profile of BSE in Portuguese cattle to that described in British cattle. *Vet Rec* 2000, **147**, 486-488.
12. Parchi P., Capellari S., Chen S.G., Petersen R.B., Gambetti P., Kopp N., Brown P., Kitamoto T., Tateishi J., Giese A., Kretzschmar H.: Typing prion isoforms. *Nature* 1997, **386**, 232-233.
13. Patterson W.J., Painter M.J.: Bovine spongiform encephalopathy and new variant Creutzfeldt-Jakob disease: an overview. *Commun Dis Public Health* 1999, **2**, 5-13.

14. Stack M.J., Chaplin M.J., Clark J.: 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* 2002, **104**, 279-286.
15. Sweeney T., Kuczius T., McElroy M., Parada M.G., Groschup M.H.: Molecular analysis of Irish sheep scrapie cases. *J Gen Virol* 2000, **81**, 1621-1627.