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Atypical Proteinase K-Resistant Prion Protein (PrP^{res}) Observed in an Apparently Healthy 23-Month-Old Holstein Steer

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Since October 18, 2001, 'bovine spongiform encephalopathy (BSE) examination for all cattle slaughtered at abattoirs in the country' has been mandated in Japan by the Ministry of Health, Labour and Welfare (MHLW). 'Plateria' ELISA-kit (Bio-Rad Laboratories, Hercules, Calif., USA) is routinely used at abattoirs for detecting proteinase K (PK)-resistant prion protein (PrP^{Sc}) in the obex region. Samples positive according to the ELISA screening are further subjected to Western blot (WB) and histologic and immunohistochemical examination (IHC) at the National Institute of Infectious Diseases (NIID) or Obihiro University. If PrP^{Sc} is detected either by WB or by IHC, the cattle are diagnosed as BSE. The diagnosis is approved by the Expert Committee for BSE Diagnosis, MHLW. From October 18, 2001 to September 30, 2003, approximately 2.5 million cattle were screened at abattoirs. A hundred and ten specimens positive according to ELISA were subjected to WB/IHC. Seven showed positive by both WB and IHC, all exhibiting the typical electrophoretic profile of a high content of the di-glycosylated molecular form of PrP^{Sc} (1-3) and the distinctive granular deposition of PrP^{Sc} in neuronal cells and neuropil of the dorsal nucleus of vagus.

An ELISA-positive specimen from a 23 month-old Holstein steer slaughtered on September 29, 2003, in Ibaraki Prefecture (Ibaraki case) was sent to the NIID for confirmation. The animal was reportedly healthy before slaughter. The OD titer in ELISA was slightly higher than the 'cut-off' level given by the manufacturer. The histology showed no spongiform changes and IHC revealed no signal of PrP^{Sc} accumulation typical for BSE. However, WB analysis of the homogenate that was prepared from the obex region and used for ELISA revealed a small amount of PrP^{Sc} with an electrophoretic profile different from that of typical BSE-associated PrP^{Sc} (1-3). The characteristics were (i) low content of the di-glycosylated molecular form of PrP^{Sc}, (ii) a faster migration of the non-glycosylated form of PrP^{Sc} on SDS-PAGE, and (iii) less resistance against PK digestion as compared with an authentic PrP^{Sc} specimen derived from an 83-month-old Holstein (Wakayama case) (Fig. 1). Table 1 summarizes the relative amounts of three distinctive glycoforms (di-, mono,

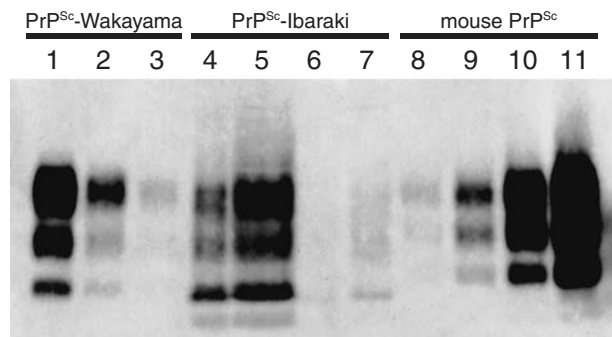


Fig. 1. Western blot analysis of PrP^{Sc} after proteinase K digestion. Lanes 1-3: typical bovine PrP^{Sc} obtained from Wakayama case (32, 8 and 2 μ g tissue equivalent). Lanes 4 and 5: Ibaraki case PrP^{Sc} (2.5 and 10 mg tissue equivalent). Lanes 6 and 7: Ibaraki case PrP^{Sc} after additional proteinase K (PK) digestion (2.5 and 10 mg tissue equivalent). Lanes 8-11: mouse PrP^{Sc} (0.1, 0.4, 1.5 and 6 μ g tissue equivalent). Western blot analysis was performed according to the protocol recommended by the Expert Committee for BSE Diagnosis, MHLW. Briefly, 50 mg of brain tissue was hydrolyzed successively with collagenase (50 μ g/ml, for 30 min) and PK (40 μ g/ml, for 30 min) at 37°C in 500 μ l of 50 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl, 2% zwittergent 3-14, 0.5% sarcosyl and 5% 2-butanol. After PK was inhibited by the addition of Pefa-block (2 mM), the homogenate was hydrolyzed with DNase I (40 μ g/ml) for 5 min at room temperature. PrP^{Sc} was then precipitated by the addition of 250 μ l of 2-butanol-methanol mixture (5:1, v:v) and centrifugation at 15,000 rpm for 15 min. The precipitates were dissolved with 50 μ l of the SDS-sample buffer and SDS-PAGE (12% polyacrylamide gel was applied) (lanes 1-5 and 8-11). Alternatively, the precipitates thus obtained were subjected to the second round of PK digestion before applying to SDS-PAGE (lanes 6, 7). Proteins were transferred onto PVDF membrane and PrP^{Sc} was detected by a mouse monoclonal antibody 44B1 (mAb 44B1), which recognizes a discontinuous epitope located between 155 and 231 amino acids in the mouse PrP sequence (Kim, C-L. et al., Virology, in press), and horse-radish peroxidase-labeled anti-mouse IgG for ECL-chemiluminescence (Amersham, Buckinghamshire, UK) detection. Upon the WB described above, PrP^{Sc} contained in as small as 1-2 μ g brain tissues (obex or thalamus) of BSE affected cattle (lane 3) and PrP^{Sc} in 0.1 μ g brain tissue of terminally sick mice (lane 6) were detectable.

non-glycosylated) of PrP^{Sc} calculated by densitometric analysis of the blot shown in Fig. 1. As 2.5 mg wet weight obex-equivalent homogenate of the Ibaraki case (Fig. 1, lane 4) gave slightly stronger band intensities of PrP^{Sc} than an 8 μ g wet weight obex-equivalent homogenate of a typical BSE-affected Wakayama case (Fig. 1, lane 2), the amount of PrP^{Sc}

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Table 1. Comparison of the glycoform ratio between typical and atypical PrP^{Sc}

Molecular species in PrP ^{Sc}	Ratio of the band intensity (%)	
	Wakayama case (typical case)	Ibaraki case (atypical case)
Di-glycosylated PrP ^{Sc}	69.3	47.6
Mono-glycosylated PrP ^{Sc}	23.2	26.5
Non-glycosylated PrP ^{Sc}	7.5	25.9

The band intensities of the non-, mono-, and di-glycosylated forms of PrP^{Sc} of the Wakayama and Ibaraki cases (Fig. 1, lanes 1 and 5) were determined by a digital-image analysis software (Image Gauge, version 3.45, Fuji Photo Film Co., Tokyo), and the ratio of the three glycoforms were calculated.

accumulated in the Ibaraki case was calculated to be 1/500-1/1000 of the Wakayama case. In the Ibaraki case, the PrP^{Sc} bands were not detectable in the homogenates of the proximal surrounding region of the obex. These findings were consistent with the low OD value in ELISA, i.e., 0.2-0.3 for the Ibaraki case versus over 3.0 for the Wakayama case. The DNA sequence of the PrP coding region of the Ibaraki case was the same as that appearing in the database (GenBank accession number: AJ298878). More recently, we encountered another case that resembled the Ibaraki case. It was a 21-month-old Holstein steer from Hiroshima Prefecture. WB showed typical BSE-specific PrP^{Sc} deposition though IHC did not detect positive signals of PrP^{Sc} (data not shown).

Though the clinical onset of BSE is usually at around 5 years of age or later, a 20-month-old case showing the clinical signs has been reported (4). Variant forms of BSE similar to our cases, i.e., with atypical histopathological and/or biochemical phenotype, have been recently reported in Italy (5) and in France (6). Such variant BSE was not associated with mutations in the prion protein (PrP) coding region as in our case (5,6).

The Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) announced a ban of feeding ruminants with meat bone meal (MBM) on September 18, 2001, and a complete ban was made on October 15 of the same year. According to the recent MAFF report, the previous seven cases of BSE in Japan were cattle born in 1995-1996 and

possibly fed with cross-contaminated feed. However, the two cattle in this report were born after the complete ban. Whether contaminated MBM was implicated in the present cases remains to be investigated.

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