

## Short Communication

# Experimental Transmission of Two Young and One Suspended Bovine Spongiform Encephalopathy (BSE) Cases to Bovinized Transgenic Mice

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**SUMMARY:** Bovine spongiform encephalopathy (BSE) is caused by a prion that primarily consists of an abnormal isoform of the prion protein (PrP<sup>Sc</sup>). Since PrP<sup>Sc</sup> is partially resistant to proteolytic digestion, the routine diagnosis of BSE is based on the immunological detection of the proteinase K (PK)-resistant moiety of PrP<sup>Sc</sup> (PrP<sup>core</sup>). However, transmission studies are indispensable in order to demonstrate prion infectivity and to analyze prion characteristics. Transmission experiments were accordingly performed on 2 young BSE cases (BSE/JP8, BSE/JP9) and 1 suspected BSE case (Suspended-1) that were detected by the BSE screening program in Japan. In this study, we attempted to transmit the prion from these 3 animals by using transgenic mice overexpressing bovine PrP (TgBoPrP). In spite of the use of BSE-sensitive transgenic mice, none of the mice developed neurological signs nor accumulated PrP<sup>Sc</sup> in their brains for more than 600 days post-inoculation, even with subsequent blind passages. The results of a dilution experiment using the classical BSE prion indicated that prion infectivity in these 3 cattle was below the detection limit of 10<sup>3.0</sup> LD<sub>50</sub>/g.

Bovine spongiform encephalopathy (BSE) is a fatal neurological disease in cattle that was first recognized in the United Kingdom in 1986 (1). The disease belongs to a group of transmissible spongiform encephalopathies (TSEs), or prion diseases (2). The occurrence of the BSE epizootic could be the result of consumption of a BSE prion contaminating proprietary concentrates or feed supplements. Recent cases of BSE have been reported throughout most of Europe, North America, and Japan.

The experimental transmissibility of BSE to cattle and to other animals has been previously demonstrated; however, the nature of the TSE agents has not been fully elucidated. A misfolded isoform of the prion protein (PrP), designated PrP<sup>Sc</sup>, is considered to be responsible for these diseases. This PrP<sup>Sc</sup> forms the main component of the prion, and it is partially resistant to proteinase digestion. Several commercial kits are available for BSE diagnosis, and most of which are based on the immunological detection of the proteinase-resistant moiety of PrP<sup>Sc</sup> (PrP<sup>core</sup>). The detection of accumulated PrP<sup>Sc</sup> by immunohistochemistry (IHC) and that of PrP<sup>core</sup> by Western blot (WB) analysis are routinely performed as confirmatory tests.

The uniform pathology among BSE-affected cattle and the limited results obtained after BSE transmission experiments were conducted in mice have led to the assumption that BSE is caused by a single prion strain (classical BSE). Recently, however, different phenotypes have been reported among BSE cases (atypical BSE) in Japan, Europe, and North America, and the transmissibility of certain atypical BSE cases has been confirmed (3,4). Atypical BSE cases are currently classified into at least two groups, namely, the L-type and the H-type group, in accord with the molecular weight of PrP<sup>core</sup>. Sheep

scrapie prions have been classified into various strains based on their varying incubation periods and/or differences in the lesion profile of spongiform changes observed in inbred mice. The molecular basis for strain variation remains unclear; however, according to the "protein-only" hypothesis, strain characteristics are encoded within different conformations of PrP<sup>Sc</sup>.

Between September 2001 and March 31, 2007, the presence of BSE infection was confirmed in 32 cattle in Japan as a result of the BSE screening and surveillance programs conducted by the Ministry of Health, Labour and Welfare (MHLW) and the Ministry of Agriculture, Forestry and Fisheries (MAFF), respectively (5). Most of the BSE-diagnosed cattle exhibited a heavy accumulation of PrP<sup>core</sup> or PrP<sup>Sc</sup> in the brain, as confirmed by both WB and IHC. Samples from two young healthy Holstein steers yielded weakly positive primary enzyme-linked immunosorbent assay (ELISA) (Platelia BSE; Bio-Rad, Hercules, Calif., USA) results, and revealed an accumulation of unusually small amounts of PrP<sup>core</sup> detected by WB; the amount of PrP<sup>core</sup> in the brains of the affected animals was estimated to be as low as 1/1,000 of that in a classical BSE case (BSE/JP6), as estimated by WB (6). Interestingly, one of the cattle (BSE/JP8) accumulated a distinct PrP<sup>core</sup> with a different glycoform profile and with proteinase K (PK)-resistance properties that differed from those of the classical BSE case, as revealed by WB; this case was classified as atypical BSE (7). In addition to these two young steers, we also detected a faint PrP<sup>core</sup>-like signal by WB in a sample obtained from an ELISA-weakly positive, 20-year-old Japanese Black cow (Suspended-1). However, due to the faint signal, the diagnosis of this case was equivocal. The specific details regarding these three cattle are listed in Table 1.

No spongiform changes and/or PrP<sup>Sc</sup> deposition were observed in any of these three cases by histopathological examination. In all three cases, the PrP<sup>core</sup> accumulation was limited to the obex, which was examined by means of ELISA; it was

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not possible to perform a detailed analysis of the remaining brain tissues. Thus, in these cases, we attempted to amplify PrP<sup>Sc</sup> by using bovine PrP-overexpressing transgenic (TgBoPrP) mice with a null background (kindly provided by Dr. S. B. Prusiner) (8). It has been reported that these mice are 10 times more sensitive than cattle and 1,000 times more sensitive than RIII mice to infection with BSE prions (9). In order to determine the sensitivity of the bioassay using TgBoPrP mice, the brain homogenates of BSE cattle (classical BSE; provided by VLA, Weybridge, UK) were serially diluted, and 20  $\mu$ l of the dilution were intracerebrally inoculated into the mice. On alternate days, these animals were monitored for the devel-

opment of clinical signs and stability of health. The mice that were inoculated with the homogenate from the classical BSE infection displayed clinical signs such as behavioral changes, weight loss, and hind limb paresis; the mice that did not exhibit these clinical signs were cared for until they died of natural causes. The results of the end-point titration experiment with classical BSE (97/04417) are shown in Table 2. The infectivity titer for the examined BSE was determined to be 10<sup>6.7</sup> LD<sub>50</sub>/g. Since incubation period is correlated with prion titer (10), an infective titer of 10<sup>5.2</sup> LD<sub>50</sub>/g was estimated for the BSE/JP6 sample, the brain homogenate of which was used as an internal standard for the comparison of WB signal intensities. All mouse samples used in this experiment were subjected to WB to determine the presence of PrP<sup>core</sup> in the brain. Brain homogenate preparation and electrophoresis were performed as described in our previous paper (11). The blotted membrane was incubated with anti-PrP T2 monoclonal antibody (mAb) (12), and the signals were detected using a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Inc., Rockford, Ill., USA). PrP<sup>Sc</sup> deposition was also examined by IHC.

We attempted to transmit the disease from the three cattle (BSE/JP8, BSE/JP9, and Suspended-1) to the TgBoPrP mice. Due to limitations of the available sample, we used the remaining homogenates for the primary ELISA test (homogenate for the grinding buffer of the Platelia BSE kit). The homogenate was diluted four times with phosphate-buffered saline (PBS; final concentration, 5% [w/v]) and was used for the transmission study. As shown in Table 2, none of the TgBoPrP mice that were intracerebrally inoculated with

Table 1. Summary of examined BSE cases

Case	Age of cattle	ELISA titer <sup>1)</sup> (cut off)	WB	IHC	Spongiform changes
BSE/JP8 <sup>2)</sup>	23 months	0.20, 0.21 (0.23)	+	-	-
BSE/JP9 <sup>3)</sup>	21 months	0.29, 0.29 (0.24)	+	-	-
Suspended-1 <sup>4)</sup>	20 years	0.43, 0.44 (0.23)	±	-	-
BSE/JP6 <sup>5)</sup>	83 months	3.1, 3.3 (0.23)	+	+	+

<sup>1)</sup>: Optical density value of Platelia BSE (Bio-Rad).

<sup>2)</sup>: Young, atypical BSE case (7), <http://www.mhlw.go.jp/houdou/2003/10/h1006-2.html> (in Japanese).

<sup>3)</sup>: Young, classical BSE case, <http://www.mhlw.go.jp/houdou/2003/11/h1104-3.html> (in Japanese).

<sup>4)</sup>: <http://www.mhlw.go.jp/houdou/2003/03/h0327-2.html> (in Japanese).

<sup>5)</sup>: Classical BSE case (6).

Table 2. Transmission of BSE to TgBoPrP mice

A. Titration assay of classical BSE<sup>1)</sup>

Inoculum	No. diseased/ no. inoculated	Mean $\pm$ SD (days)
ori	6/6	217.8 $\pm$ 3.8
10 <sup>-1</sup>	6/6	257 $\pm$ 2.6
10 <sup>-2</sup>	6/6	309 $\pm$ 53.4
10 <sup>-3</sup>	6/6	386 $\pm$ 13.9
10 <sup>-4</sup>	3/6	479 $\pm$ 131.0
10 <sup>-5</sup>	0/6	>495

B. Transmission study of Japanese BSE cases

Inoculum	Mice	No. diseased/ no. inoculated	Mean $\pm$ SD (days) or sacrificed days
BSE/JP8 <sup>2)</sup>			
Primary passage	TgBoPrP	0/5	(600, 786, 788, 788, 860)
2nd passage	TgBoPrP	0/7	>550
2nd passage	ICR	0/7	>550
BSE/JP9 <sup>3)</sup>			
Primary passage	TgBoPrP	0/6	(505, 577, 704, 881, 927, 927)
2nd passage	TgBoPrP	0/7	>495
2nd passage	ICR	0/7	>495
Suspended-1 <sup>4)</sup>	TgBoPrP	0/7	(717, 811, 831, 864, 864, 892, 927)
PBS	TgBoPrP	0/5	(432, 475, 534, 609, 717)
BSE/JP6 <sup>5)</sup>	TgBoPrP	5/5	277.2 $\pm$ 12.2

All mice were tested for the presence of PrP<sup>core</sup> in the brain by WB, and mice that were positive were considered to be diseased.

<sup>1)</sup>: BSE sample obtained from the UK. Infectivity titer per gram was 10<sup>6.7</sup>.

<sup>2)</sup>: Case of atypical BSE in a 23-month-old Holstein steer.

<sup>3)</sup>: Case of classical BSE in a 21-month-old Holstein steer.

<sup>4)</sup>: BSE diagnosis was suspended in a 20-year-old Japanese Black. The faint unusual PrP<sup>core</sup>-like band was observed by WB; however, no spongiform change or PrP<sup>Sc</sup> deposition were detected in pathology.

<sup>5)</sup>: Case of classical BSE in a 83-month-old Holstein cow.

the brain homogenates obtained from the two young BSE cases and from the one suspended case displayed any clinical signs associated with BSE. The brain homogenate obtained from TgBoPrP mice that died without clinical signs (600 and 788 days post-inoculation in BSE/JP8 and 505 days post-inoculation in BSE/JP9) were intracerebrally inoculated into additional TgBoPrP mice for a second passage. When observed 500 days post-inoculation, no clinical signs and/or abnormalities were observed after the second passages in these mice. The mice inoculated with PBS, the negative control, died of natural causes between 432 and 717 days post-inoculation.

PrP<sup>core</sup> was detected in the mouse brains inoculated with BSE/JP6, and its glycoform and molecular size were similar to that of classical BSE (Fig. 1, lanes 5-7). A PrP signal at 25-kD was detected in the PK-treated brain homogenate of some of the mice inoculated with material from both young and suspected BSE cases, although as shown in Fig. 1, the glycoform profile different from that of PrP<sup>core</sup> in the original inoculum (7, <http://www.mhlw.go.jp/houdou/2003/11/h1104-3.html>) (Fig. 1, lanes 1-3). The 25-kD signal was not observed in the brains of any young mice examined (6 months old) (lane 4). This observation suggests that the PK-resistant PrP detected in the brains of the older TgBoPrP mice was a product of spontaneous protein misfolding. It was of note that this 25-kD band was also generated *in vitro*, as determined by protein misfolding cyclic amplification (PMCA) from the normal cattle and mouse brain homogenates; this 25-kD product was designated as PrP<sup>C-res</sup> (13). Recently, we confirmed that the PrP<sup>C-res</sup> in old TgBoPrP mice was not converted to PrP<sup>Sc</sup> (data not shown). Furthermore, no PrP<sup>Sc</sup> signal was detected by IHC in the brains of older TgBoPrP mice (data not shown), and no transmissibility was observed in either TgBoPrP or ICR mice (Table 2). Thus, PrP<sup>C-res</sup> may be the result of overexpression of PrP<sup>C</sup> in TgBoPrP mice. It appeared that the 25-kD band was not a self-propagating product of the prion in the inoculum.

It has been reported that some mice inoculated with a BSE sample did not accumulate PrP<sup>Sc</sup> in their brains, and that PrP<sup>Sc</sup> appeared during serial passages following adaptation to the new host (14). We accordingly examined the secondary passage of the two young BSE cases (BSE/JP8 and BSE/JP9) to TgBoPrP and wild-type mice (ICR, SLC/Japan). The blind passage also revealed the non-transmissibility of these samples to the TgBoPrP and wild-type mice (Table 2).

We were unsuccessful at transmitting the three present cases to TgBoPrP mice via intracerebral inoculation. However, given the extremely low content of PrP<sup>Sc</sup> in these preparations, this does not necessarily mean that the PrP<sup>core</sup> in the inocula were not infectious. Considering the intensity of the PrP<sup>core</sup> signal on the WB, it was estimated that the amount of PrP<sup>core</sup> in the young and suspected BSE cases was as low as approximately 1/1,000 of that of BSE/JP6, for which the prion titer was  $10^{5.2}$  LD<sub>50</sub>/g ( $10^{2.2}$  LD<sub>50</sub>/mg). In this experiment, each mouse received 1 mg (20  $\mu$ l of a 5% brain homogenate) of brain tissue as an inoculum. If we assume a low content of PrP<sup>core</sup> in the preparation, the mice received only  $10^{-0.8}$  LD<sub>50</sub> (0.16 LD<sub>50</sub>) units of prion; this amount would be equivalent to the very limit of sensitivity of the bioassay.

If the susceptibility of the TgBoPrP mice to atypical BSE was similar to their susceptibility to classical BSE, then the amount of PrP<sup>core</sup> in the cattle brain might have been below the bioassay detection limit. Clearly, the precise susceptibility of the TgBoPrP mice to atypical BSE remains uncertain. The infectivity of BSE/JP8 that is classified as atypical BSE (Table

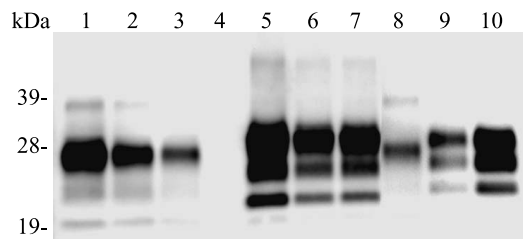


Fig. 1. WB analysis of the brain of the BSE inoculated TgBoPrP mice. Mice brain was homogenized (10%) in detergent buffer and incubated with 50  $\mu$ g/ml of PK for 30 min at 37°C. The sample was mixed with an equal volume of sodium dodecyl sulfate (SDS) sample buffer and then subjected to WB (11). Lanes 1-3, TgBoPrP mice inoculated with young atypical BSE (BSE/JP8) (860 days post-inoculation); lane 4, mock-infected TgBoPrP mice (6 months old); lanes 5-7, TgBoPrP inoculated with classical BSE (BSE/JP6); lane 8, mock-infected aged TgBoPrP mice (800 days old); and lanes 9-10, PrP<sup>core</sup> from scrapie-infected mouse brain (0.4 and 1.6  $\mu$ g brain equivalents). Lanes 1 and 5, 125  $\mu$ g; lanes 2 and 6, 50  $\mu$ g; lanes 3 and 7, 25  $\mu$ g; lanes 4 and 8, 250  $\mu$ g brain equivalents were examined per lane. Mab.T2 was used for detection.

2) should be carefully considered in this context. It has been reported that the L- and H-types of atypical BSE have different incubation periods in TgbovXV mice (4). This result indicated that several different atypical BSE strains may exist, and that TgBoPrP mice might be less sensitive to certain atypical BSE strains than to the typical strain. Thus, in order to investigate atypical BSE prions, it will be necessary to develop an experimental animal with high susceptibility to atypical BSE prions.

Most of the atypical BSE cases reported thus far have been considered to be sporadic rather than genetic (4,15). None of the cattle examined in this study harbored the amino acid substitution associated with the PrP gene (Hagiwara et al., unpublished data). The two young cattle with BSE were born in 2001-2002, immediately after the animal feed ban was enforced in Japan following the first reported occurrence of BSE. Therefore, we believe that these cases were a result of cross-contamination of the remaining feed.

Recently, another atypical BSE infection in an old cow (14 years old) was reported in Japan (BSE/JP24); a different phenotype of PrP<sup>core</sup> was observed by WB, and PrP<sup>Sc</sup> deposition in this case was detected by IHC (16). Precautions must be taken to account for the possibility of sporadic BSE occurrence in old cattle such as the 20-year-old Suspended-1 and the 14-year-old atypical BSE animals. Unfortunately, the lack of evidence renders it difficult to evaluate the risk of atypical BSE infection in humans.

Along these lines, it is important to consider the sensitivity of the WB technique applied in the BSE screening and active surveillance programs in Japan (<http://www.mhlw.go.jp/english/topics/foodsafety/bse/dl/3-1-2-1.pdf>). Common practice dictates the detection of PrP<sup>core</sup> contained in 1  $\mu$ g of brain tissue of BSE/JP6 confirmed by WB as employed in these programs; thus, the sensitivity of WB is similar to that of the bioassay system using TgBoPrP mice. A highly sensitive WB that can detect small amounts of PrP<sup>core</sup> would be an extremely advantageous tool for detecting BSE-affected cattle still in the incubation period.

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