Original Article

Neuroanatomical Distribution of Disease-Associated Prion Protein in Experimental Bovine Spongiform Encephalopathy in Cattle after Intracerebral Inoculation

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SUMMARY: The pathologic disease-associated prion protein (PrPSc) has been shown to be expressed in the central nervous system (CNS) of Holstein cattle inoculated intracerebrally with 3 sources of classical bovine spongiform encephalopathy (BSE) isolates. Several regions of the brain and spinal cord were analyzed for PrPSc expression by immunohistochemical and Western blotting analyses. Animals euthanized at 10 months post-inoculation (mpi) showed PrPSc deposits in the brainstem and thalamus, but no vacuolation; this suggested that the BSE agent might exhibit area-dependent tropism in the brain. At 16 and 18 mpi, a small amount of vacuolation was detected in the brainstem and thalamus, but not in the cerebral cortices. At 20 to 24 mpi, when clinical symptoms were apparent, heavy PrPSc deposits were evident throughout the brain and spinal cord. The mean time to the appearance of clinical symptoms was 19.7 mpi, and the mean survival time was 22.7 mpi. These findings show that PrPSc accumulation was detected approximately 10 months before the clinical symptoms of BSE became apparent. In addition, the 3 sources of BSE prion induced no detectable differences in the clinical signs, incubation periods, neuroanatomical location of vacuoles, or distribution and pattern of PrPSc depositions in the brain.

INTRODUCTION

Bovine spongiform encephalopathy (BSE), a type of transmissible spongiform encephalopathy (TSE), is a fatal neurodegenerative disease affecting cattle. The disease was first identified in the United Kingdom (UK) in 1986 (1); subsequently, it spread to European, Asian, and North American countries. The first case of BSE in Japan was reported in September 2001 (2), and the most recent case, the 36th, was confirmed in January 2009. BSE is characterized by spongiform changes (3) and accumulation of the disease-associated prion protein (PrPSc) in the central nervous system (CNS) (4). PrPSc is commonly accepted as the pathological agent of BSE and is thought to be a post-translationally modified form of the host-encoded membrane glycoprotein (PrPC) (5). PrPSc is the only known disease-specific marker (6,7).

The pathological agent of BSE is transmissible to different mammalian species. A variant form, i.e., the degenerative brain disease Creutzfeldt-Jakob Disease (vCJD) has been reported in the UK and several other countries, and it is thought that this disease is caused by the consumption of BSE-contaminated beef products (8–11). Therefore, it is important to understand the pathogenesis of BSE in cattle in order to eliminate BSE-contaminated food from human food and thereby preserve public health.

The uniformity in the pathological features and biochemical profile of the proteinase K (PK)-resistant PrPSc (PrPres) in BSE-affected cattle suggests that a single prion strain is responsible for BSE in these animals. Recently, variants of BSE (named atypical BSE) have been detected in cattle in Europe (12,13), North America (14,15), and Japan (16,17). Currently, atypical BSE cases are classified into 2 groups; those expressing PrPres of lower (L-type BSE) molecular weight and those expressing PrPres with higher (H-type BSE) molecular weight than the PrPres of classical BSE (C-BSE) (18).

Our current knowledge of the pathogenesis of C-BSE in cattle is based on the examination of tissues obtained from orally infected cattle that have been euthanized at different stages of the disease. A mouse bioassay of infectivity showed that in cattle, the infection was limited to the brain, spinal cord, eyes, dorsal root ganglia, and distal ileum (19–21). Occasionally, infectivity has also been detected in the bone marrow and tonsils of experimentally infected cattle (22,23), and recent studies have shown that peripheral tissues other than the CNS may harbor PrPSc at the clinical stages of the disease (24,25). The distribution of PrPSc in the brain has been
mapped in both naturally occurring C-BSE and orally infected cattle (26–28). However, it would be of interest to evaluate the relationship between the time of detection of PrPres in the CNS and the clinical course of the disease. For this purpose, we used the intracerebral inoculation route to infect cattle with 3 different C-BSE strains—one isolated in Kanagawa, Japan (BSE/JP5); one, in Wakayama, Japan (BSE/JP6) (29); and one, in the UK (BSE/UK) (30). We then proceeded to measure the distribution of PrPres in the CNS of the infected animals by immunohistochemical and Western blotting analyses.

MATERIALS AND METHODS

Ethical considerations: All experiments involving animals were approved by the Animal Ethical Committee and the Animal Care and Use Committee of both Hokkaido Animal Research Center and National Institute of Animal Health.

Inoculation of cattle with C-BSE agents: Brain homogenates (10% w/v) were prepared from the brains of 3 cattle: one had been naturally infected with C-BSE (BSE/UK) provided by the Veterinary Laboratory Agency, UK; one was infected with domestic Kanagawa-1 (BSE/JP5) at 80 months of age; and one was infected with domestic Wakayama (BSE/JP6) at 83 months of age (29,30). Sixteen female Holstein calves aged 3 months were used for this experiment (n = 8 for BSE/UK, n = 4 for BSE/JP5, and n = 4 for BSE/JP6) (Table 1). Each animal was inoculated in the right side of the midbrain and 1 mL of brain homogenate was withdrawn from the brain by using an 18-gauge 7-cm disposable needle (NIPRO, Osaka, Japan). Two uninfected cattle served as controls and were euthanized at 27 months of age.

Neuropathology: At necropsy, the brains and cerebellum were removed and hemisected at the midline. Samples of various tissues were fixed in 10% neutral buffered formalin (pH 7.4) for 3 days at 37°C, including those of the left hemisphere and spinal cord at the levels of cervical (C8) and lumbar enlargement (L6). The contralateral side was frozen at −80°C for Western blotting analysis of PrPres. Coronal slices of the brain and various tissues were cut at 3–4 mm thickness and placed in plastic cassettes, which were immersed in 98% formic acid for 60 min at room temperature (RT) to reduce infectivity (31). The tissues were automatically processed through a graded series of alcohol to xylene and then paraffin-embedded (ETP-150C; Sakura Finetek Japan, Tokyo, Japan). Serial sections were cut at a thickness of 4 μm, mounted on silane-coated glass slides (New Silane II; Muto Pure Chemicals Co., Tokyo, Japan) and stained with hematoxylin and eosin or processed for immunohistochemistry, as described below. The distribution and extents of vacuolation in the brain were scored according to the method described by Simmons et al. (32). A vacuolation lesion profile was created by plotting the mean vacuolation score for each neuroanatomical area against the assigned code for that area.

PrPres immunohistochemistry: For each animal, tissue samples were examined from at least 8 areas of the brain and 2 spinal cord levels: frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, and cerebellum, and the C8 and L6 levels of the spinal cord. The paraffin-embedded tissue sections were pretreated at RT for PrPres antigen retrieval using a recently developed chemical method (33). Briefly, deparaffinized and rehydrated tissue sections were immersed in a bath of 98% formic acid for 5 min, incubated with 0.5% (w/v) potassium permanganate (in 0.1 M phosphate buffer, pH 7.0) for 10 min, and then washed in distilled water 3 times. The sections were soaked in 1% sodium sulfite for 2 min and then washed in distilled water. The slides were then immersed in a solution of 0.1% N-lauroylsarcosine, 75 mM sodium hydroxide, and 2% sodium chloride for 10 min. Next, the sections were washed in tap water for 5 min and then placed in an immunohistochemical autostainer (Dako Cytomation Autostainer Universal Staining System; Dako, Carpinteria, Calif., USA). They were then incubated sequentially with 1 μg/mL anti-PrP primary monoclonal

<table>
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<tr>
<th>Case</th>
<th>Code</th>
<th>Inoculum</th>
<th>Time of clinical onset (mpi)</th>
<th>Clinical signs at onset</th>
<th>Terminal clinical signs</th>
<th>Time at necropsy (mpi)</th>
<th>Spongiform change</th>
<th>PrPres by IHC</th>
<th>PrPres by WB</th>
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<td>4612</td>
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<td>22 abnormal posture</td>
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<td>+</td>
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</table>

BSE, bovine spongiform encephalopathy; mpi, months post-inoculation; IHC, immunohistochemistry; WB, Western blotting.
antibody (mAb) T1, goat anti-mouse Fab’ universal immunoperoxidase dye (Histofine SimpleStain MAX-PO (M); Nichirei, Tokyo, Japan), and 3–3’ diamino benzidine tetrachloride as the chromogen. The T1 primary mAb was raised against mouse PrP amino acid residues 121–231 and cross-reacts with bovine PrP (34). Finally, the sections were counterstained with hematoxylin. All the steps in the immunohistochemical staining procedure were carried out at RT.

**Immunohistochemical PrPSc mapping and profiling:** For each animal, the topographical distribution of PrPSc deposition was mapped at 14 different areas of the CNS: frontal cortex, temporal cortex, parietal cortex, occipital cortex, striatum, hippocampus, thalamus, midbrain, pons, medulla oblongata at the obex, cerebellar cortex, cerebellar medulla, and spinal cord at C8 and L6 segments. The PrPSc were classified into 8 types, as previously published (27,35). Intracellular PrPSc were subdivided into intraneuronal and intraglial granular deposits. Reports indicate that the stellate-type of PrPSc immunolabeling in astrocytes differed from the intraglial-type labeling (27,35). Extracellular PrPSc deposits in the neuropil were classified as linear, perineuronal, fine particulate, coarse granular, and coalescing.

PrPSc accumulation was scored subjectively for intensity and extent on a scale from 0 to 4 (0, negative; 1, apparent at high magnification; 2, apparent at moderate magnification; 3, apparent at low magnification and moderate amounts of accumulation; and 4, large amounts of accumulation) (36,37); it was then topographically mapped to the different CNS areas mentioned above.

**Western blotting:** Tissue samples were obtained from 18 areas of the brain and spinal cord, as shown schematically in Fig. 1. The tissues were homogenized in a buffer containing 100 mM NaCl and 50 mM Tris-HCl (pH 7.6). The homogenate was mixed with an equal volume of buffer containing 4% (w/v) Zwittergent 3–14 (Merck, Darmstadt, Germany), 1% (w/v) Sarkosyl, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.6), and incubated with 0.25 mg collagenase, followed by incubation with PK (final concentration, 40 μg/mL) at 37°C for 30 min. PK digestion was terminated by the addition of 2 mM Pefabloc (Roche Diagnostics, Basel, Switzerland). The sample was then mixed with 2-butanol:methanol (5:1) and centrifuged at 20,000 g for 10 min. The extracts were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, Mass., USA). The blotted membrane was incubated with horse-radish-conjugated anti-PrP mAb T2 (34) at RT for 60 min. Signals were developed with a chemiluminescent substrate (SuperSignal; Pierce Biotechnology, Rockford, Ill., USA).

**RESULTS**

**Clinical signs:** Of the 16 animals studied, 7 (Cases 1–7) showed no clinical signs of BSE even as late as 19 months post-inoculation (mpi) (Table 1). The remaining 9 animals (Cases 8–16) exhibited the initial clinical signs of disease between 18 and 22 mpi (19.7 ± 1.6, mean ± standard deviation); these signs included lowering of the head, heightened anxiety, and sensitivity to auditory stimuli. Within 2 to 3 months of the appearance of the initial clinical symptoms, the animals developed ataxia of the hind limbs, which progressed to difficulty in raising them without assistance. C-BSE-infected cattle were euthanized during this stage of the disease between 20 and 24 mpi (Table 1). There was no detectable difference in the clinical signs exhibited by animals inoculated with the 3 different C-BSE isolates.

**Histopathology:** The severity of vacuolation in the...
Fig. 3. Schematic representation of PrPSc in different brain areas of BSE-challenged cattle at preclinical (Cases 4–7) and clinical stages of the disease (Cases 8–16). The severity of PrPSc deposition is scored on a semi quantitative scale as 0 = no deposition, 1 = scanty, 2 = mild, 3 = moderate, and 4 = severe, with color gradation between white and black as indicated. Topographical brain areas schematically represent 10 coronal slices at the level of (from upper left to lower right): frontal lobe, striatum, thalamus, occipital lobe, midbrain, pons, medulla oblongata at the obex, spinal cord at the cervical enlargement, spinal cord at the lumbar enlargement, and cerebellum.

Vacuolar changes of the brain were more frequent in the animals that exhibited clinical signs and were euthanized between 20 and 24 mpi (Cases 8–16) than in the 7 animals without clinical manifestations. The highest mean lesion scores were obtained for the thalamic nuclei and the neuropil of the central gray matter of the midbrain, and the lowest scores, for the caudal cerebral cortices and cerebellar cortex. Moreover, examination of the dorsal motor nucleus of the vagus nerve (DMNV) showed less characteristic vacuolar change. However, spongy change was much more severe and frequent in the trigeminal nucleus and solitary nucleus than in the other nuclei of the medulla oblongata at the obex level. Mild vacuolation was present in the neuropil of the gray matter in the spinal cords of all animals with clinical signs of the disease.

PrPSc immunohistochemistry: Figure 3 shows brain maps representing the topography and scoring of PrPSc at the frontal cortex level, striatum level, thalamus and brain was scored as described in Methods, and the resulting lesion profiles are summarized in Fig. 2. Animals euthanized at 3, 10, and 12 mpi (Cases 1–4) had no vacuolar changes in any regions of the brain. Two cattle (Cases 5 and 6) were euthanized at 16 and 18 mpi, when clinical signs were absent, and they showed a few vacuoles in the neuropil of the thalamic nuclei, hypothalamus, pontine nuclei, nucleus of the spinal tract of trigeminal nerve, and putamen (Fig. 2). However, no vacuolation was detected in the cerebral and cerebellar cortices of these animals.

One animal (Case 7) showed no clinical signs of the disease and was determined to be at the preclinical stage of disease when euthanized at 19 mpi. This animal had a moderate number of vacuoles widely distributed throughout the brain (Fig. 2); vacuolization of the neuropil was evident in the thalamic nuclei, pons, and midbrain, and less frequently, in the cerebral cortices, especially in the caudal cerebrum.
Fig. 4. Thalamus in Case 2. Intraglial (large arrow) and particulate (small arrows) PrPSc immunolabeling is detected in the dorsolateral thalamic nucleus. Immunohistochemical labeling with mAb T1.

Fig. 5. Medulla oblongata at the obex level in Case 12. Particulate and granular PrPSc depositions are obvious in the neuropil of the nucleus of the solitary tract (SN). In contrast, PrPSc accumulation is sparse in the dorsal motor nucleus of the vagus nerve (DMNV). Immunohistochemical labeling with mAb T1 and hematoxylin counterstain.

The initial tissue lesion was detected as sparse PrPSc deposits in the neuronal perikarya and neuropil of gray matter, as neuritic-particulate or granular and linear types in the nuclei of thalamus (mostly ventricular nuclei), midbrain, pons, medulla oblongata (mostly spinal trigeminal nucleus), and septal accumbens of the animal euthanized at 10 mpi (Case 2; Fig. 4). Interestingly, the intraneuronal type of PrPSc deposit was more frequent than the other types. The neuritic-particulate or granular type of deposition showed neuronal process labeling. Perineuronal labeling was also detected, but less frequently. In the 2 animals euthanized at 12 mpi (Cases 3 and 4), small amounts of particulate or granular labeling in the neuronal cells and particulate or granular neuropil labeling were often present in the gray matter of the C8 and L6 segments of the spinal cord. However, no PrPSc deposit could be detected in the brain sections of the animal euthanized at 3 mpi (Case 1).

The animals (Cases 5 and 6) that had no clinical signs and were euthanized at 16 and 18 mpi exhibited moderate amounts of intraneuronal and intraglial granular as well as particulate, linear, and coalescing neuropil labeling in the thalamus, midbrain, pons, medulla oblongata, cerebellar medulla, septal accumbens, and spinal cord. Minimal to slight PrPSc deposition was also present in the cerebral and cerebellar cortices, mostly in the frontal cortex.

Intraneuronal vacuoles were occasionally present in the brainstem and thalamic nuclei of the animal euthanized at 19 mpi (Case 7). PrPSc deposition was moderately localized in the brainstem, thalamic and septal nuclei, hypothalamus, cerebellar nuclei, and gray matter of the spinal cord, and was sparse in the rostral cerebral cortices and hippocampus. The labeling in the cerebral cortices of this animal was more apparent than that in the animals (Cases 5 and 6) euthanized at 16 and 18 mpi.

In general, the types and topographical distribution of PrPSc deposits were quite similar among the animals that showed clinical signs of the disease (Cases 8–16; Fig. 3). The different types of immunolabeled PrPSc, i.e., the particulate or granular neuropil, intraneuronal, perineuronal, glial, linear, and coalescing types, were widely distributed throughout the brain. PrPSc immunolabeling was most pronounced in the brainstem, thalamus, cerebellar white matter, and the gray matter of the spinal cord (Fig. 3). Small amounts of neuropil labeling were present in the DMNV at the level of the obex. In contrast, large amounts of PrPSc were evident in the nucleus of the solitary tract and the spinal tract nucleus of the trigeminal nerve (Fig. 5). Strong immunolabeling was conspicuous in both the cervical and lumbar segments of the spinal cord. Slight to moderate amounts of PrPSc deposits were dispersed in the cerebral and cerebellar cortices. The frontal cortex consistently showed the highest PrPSc deposition, while the lowest was noted in the occipital cortex. In the cerebellar cortex, PrPSc accumulation occurred in the granule cell layer, particularly just beneath the Purkinje cell layer.

Western blotting: A PrPres signal was not detected in the brain extracts from the calf euthanized at 3 mpi (Case 1), but a small amount of PrPres was detected in the brainstem and cerebellum of the animal killed at 10 mpi (Case 2; Fig. 6). The signal intensities of the extracts from different animals varied; for example, the signals obtained in Cases 4 and 7, in which the animals were killed at 12 and 19 mpi, respectively, were slightly stronger than those obtained in Cases 3 and 6, in which the animals were killed at 12 and 19 mpi, respectively. As seen in both Fig. 6 and Table 2, the spread of PrPres throughout the brain and spinal cord correlated with the progression of the disease. The results of the Western blotting analyses are summarized in Table 2.

DISCUSSION

The goal of this study was to investigate the accumulation of PrPSc in the brain of cattle intracerebrally inoculated with the C-BSE prion agent. Although this
Fig. 6. Western blotting of PrP\textsuperscript{Sc} in the CNS extracts from animals at the preclinical (A) or clinical stages of disease (B). Lanes are numbered according to the 18 different CNS regions shown in Figure 1. Each lane was loaded with 20 mg of tissue. Western blots were probed with mAb T2 to detect PrP\textsuperscript{Sc}. ND, not done.

Table 2. Detection of PrP\textsuperscript{Sc} by Western blotting of CNS tissue samples

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<th>Clinical</th>
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<td></td>
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<td>2</td>
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<tr>
<td>1* Frontal cortex</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 Parietal cortex</td>
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<td>-</td>
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</tr>
<tr>
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</tr>
<tr>
<td>6 Thalamus</td>
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</tr>
<tr>
<td>7 Parietal cortex</td>
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<td>-</td>
</tr>
<tr>
<td>8 White matter at level of thalamus</td>
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<tr>
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<td>10 Hippocampus</td>
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- none; +, positive; ++, strongly positive (compared to positive control of mouse scrapie-infected brain 1.6 µg tissue equivalent); ND, not done.

*Numbers correspond to the 18 different brain areas as shown in Fig. 1.
transmission route does not mimic the natural route of infection, which is most likely the ingestion of infectious material, intracerebral challenge seems to be the most efficient route for the synchronized induction of C-BSE in cattle. In line with this assumption, the incubation periods and disease durations of all the C-BSE-inoculated cattle were consistent with the findings of previous studies (38). In addition, although the number of study animals was small, the cattle inoculated with the 3 different C-BSE isolates did not differ in terms of the vacuolar lesion scores, the PrP\textsuperscript{Sc} topographical distribution, or the extent of PrP\textsuperscript{Sc} accumulation in the brain at the terminal disease stage. These results suggest that the 3 BSE strains used in this study may be identical and originate from a single infectious strain, which we denoted as the C-BSE prion.

We detected an early accumulation of PrP\textsuperscript{Sc} in the brainstem of infected animals; the reasons for this could be that the structure lies in the intracerebral inoculation path or because the brainstem is a target site for the C-BSE agent. Although the PrP\textsuperscript{Sc} detected in the brainstem could thus be attributed to residual material from the inoculation, no PrP\textsuperscript{Sc} was detected in the brainstem of the animal euthanized at 3 mpi (Case 1), either by Western blotting or by immunohistochemistry. This finding is consistent with a previous report of the experimental transmission of sheep scrapie (39). PrP\textsuperscript{Sc} might be widely distributed in a nonuniform manner from the inoculum point to other targeted brain areas, suggesting that the C-BSE prion had a strong regional tropism for the brainstem and thalamus (27). This possibility was not ruled out because we found that PrP\textsuperscript{Sc} was distributed throughout the brain and spinal cord, and not solely localized in the midbrain and cerebrum at the site of inoculation (40,41). In addition to the vacuolar lesion profiles, we found the topographical distribution of PrP\textsuperscript{Sc} in the brains of cattle with clinically evident disease to be consistent with that reported for cattle with naturally occurring BSE (27,29,35,42–45). The results described here also suggest that the accumulation and distribution of PrP\textsuperscript{Sc} in the brain correlated with the disease incubation period.

Although each C-BSE inoculum was prepared from the same brain region (brainstem) of infected cattle, we observed differences in the PrP\textsuperscript{Sc} signal intensity on Western blots between animals sacrificed at the same point after inoculation. For example, the intensity differed between Case 3 (BSE/UK) and Case 4 (BSE/JP6), wherein the animals were sacrificed at 12 mpi, and between Case 6 (BSE/UK) and Case 7 (BSE/JP6), wherein the animals were sacrificed at 18 and 19 mpi, respectively. These differences may be attributed to the low number of experimental animals used, variations in the infectivity titers of the inoculums, breeding conditions, or additional unknown factors associated with prion propagation in the brain. The vacuolar lesion scores of symptomatic animals in this study were considerably higher than those of asymptomatic animals, and they were consistent with those previously described for BSE-affected cattle that had been naturally or experimentally infected (32,38,46). According to the current models of peripheral pathogenesis in orally induced TSEs, the BSE prion most probably reaches the medulla oblongata and then spreads along the parasympathetic efferent fibers of the autonomic nerve system, i.e., the vagus nerves (47–49). In one study, cattle receiving a high-dose peroral challenge of the BSE agent showed initial deposition of PrP\textsuperscript{Sc} in the DMNV, celiac and mesenteric ganglion complex, and caudal mesenteric ganglion, as well as in the intermediolateral cell column of the spinal cord, but not in other areas, including the midbrain (26). The DMNV was also the first region of PrP\textsuperscript{Sc} deposition in the brain of cattle naturally affected by BSE (50) and those with experimental BSE induced by oral inoculation (26). Therefore, the discrepancy between the findings of our study and those reported for naturally occurring BSE with regard to the severity of vacuolar changes and PrP\textsuperscript{Sc} accumulation in the DMNV might be attributed to the different routes of infection in the individual studies.

In summary, we found that the earliest accumulation of PrP\textsuperscript{Sc} in intracerebrally inoculated cattle in the brainstem and thalamus occurred at 10 mpi, which was 10 months before the onset of clinical signs. PrP\textsuperscript{Sc} was widely distributed throughout the CNS during this preclinical period and accumulated at the target sites, mostly in the brainstem and thalamus. This study also indicated that clinical signs of the disease might appear after the appearance of vacuolar changes in the brain.

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Conflict of interest None to declare.

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