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Quantification of PrP^C in bovine peripheral tissues: Analysis in wild-type and PrP^C-deficient cattle

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Abstract. Cellular PrP (PrP^C) is necessary for bovine spongiform encephalopathy (BSE) infection. The purpose of the present experiment was the quantification of PrP^C in peripheral tissues to assess the risk of BSE infection from these tissues. The tissue distribution of PrP^C was examined by a sandwich enzyme-linked immunosorbent assay (sELISA) and histochemical analysis. PrP^C-deficient cows were used as a negative control. The sELISA revealed that the brain contained the highest PrP^C content (10.7 μg/g tissue), while other organs/tissues harbored lower amounts, in decreasing order as follows: longissimus capitis muscle, iliocostalis thoracis muscle, splenius muscle, biceps femoris muscle, triceps brachii muscle, longissimus thoracis muscle, ileum, jejunum, duodenum, colon, cecum, apex linguae, omotransversarius muscle, posterior part of the corpus linguae, anterior part of the corpus linguae and radix linguae (5.2- to 31-fold less PrP^C than the brain). In the tissue/organs of PrP-deficient cows, PrP^C levels were under the limit of detection. Histochemical analysis showed that PrP^C was expressed in nerve cells in intestinal tissues. The presence of PrP^C in the bovine tongue, skeletal muscles and intestines raises the possibility of PrP^{Sc} accumulation in these tissues, indicating that these organs/tissues may serve as potential sources of BSE infection.

Introduction

The cellular isoform of prion protein (PrP^C), a glycoprotein with a glycosylphosphatidyl inositol (GPI) anchor, plays a pivotal role in the infection of transmissible spongiform encephalopathies (TSEs). These comprise a family of neurodegenerative disorders affecting both humans and animals. TSEs include bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy (TME), feline spongiform encephalopathy (FSE), sheep scrapie, iatrogenic Creutzfeldt-Jakob disease (CJD), sporadic CJD (sCJD) and variant CJD (vCJD). TSEs are characterized by the accumulation of a modified form or a protease-resistant form (PrP^{Sc}), derived from PrP^C. Although the conversion of host-encoded PrP^C is crucial for disease transmission and PrP^{Sc} formation (1,2), the mechanism(s) of prion transmission remains unresolved. Furthermore, PrP^{Sc} accumulation and PrP^C deficiency induced by prion infection in the brain are presumed to be causative factors of prion disease (3). One emerging hypothesis advocates that the abovementioned conversion phenomenon occurs at the site where the infectious agent binds with PrP^C. In cattle, apart from accumulating preferentially in specified-risk materials (SRM) such as the brain and spinal cord, PrP^{Sc} was also deposited in the ischiadic nerve, tibial nerve and adrenal gland of a 94-month-old BSE-positive (11th) case in Japan (7). Thus, PrP^{Sc} has the potential to accumulate in peripheral tissues as well as in SRM. PrP^{Sc} was recently detected in the muscles of sheep (4,5), mice infected with scrapie (6), BSE or vCJD (8), hamsters infected with scrapie (9-11), TME (9,12) or BSE (8), and humans infected with sCJD (13,14). To assess the risk of PrP^{Sc} accumulation in peripheral tissues, it is important to understand the distribution and expression levels of PrP^C in these tissues. To date, although studies have been performed in rodents (15-18), sheep (19,20), cervidae (21) and humans (22), PrP^c levels in the peripheral tissues of cattle, which are regarded as transmission carriers of vCJD in humans, remain to be determined.

In this study, the anti-prion protein (PrP) monoclonal antibodies (mAbs) T2 and T17 (purified from hybridoma supernatants) were labeled with horseradish peroxidase (HRP) before being subjected to a novel sandwich enzyme-linked immunosorbent assay (sELISA) established using these mAbs. Quantification of PrP^C levels in the bovine tongue, skeletal muscles and intestines was carried out by comparison with levels in PrP gene-deficient cows.

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Key words: prion protein, sandwich ELISA, muscle, risk assessment, intestine, prion protein-deficient cow

Materials and methods

Animals and tissue materials. Fifty-nine-month-old and 6-month-old Holstein cattle were housed at the Animal Resource Center, Graduate School of Agriculture and Life Science, the University of Tokyo. All animals were treated in accordance with procedures authorized by the Animal Experiment Committee of the School of Agriculture and Life Science. After the animals were anesthetized with propofol and euthanized by exsanguination, the brain, apex linguae, anterior part of the corpus linguae, posterior part of the corpus linguae, radix linguae, splenius muscle, longissimus capitis muscle, omotransversarius muscle, longissimus thoracis muscle, iliocostalis thoracis muscle, triceps brachii muscle, biceps femoris muscle, colon, duodenum, ileum, jejunum and cecum were isolated. The liver was removed from 59-month old Holestein cattle. The brain of an 8-week-old Syrian hamster (Japan SLC, Inc.) was isolated and used as a reference.

This study also examined and compared each tissue (muscle, ileum and liver) from prion gene-deficient cows (n=2) as a negative control for prion protein, kindly provided by JA ZEN-NOH. The cattle lacking prion protein were produced using methods reported previously (23).

Recombinant bovine PrP. Recombinant bovine PrP (25-241) (Alicon, Schlieren, Switzerland) was used in this study.

Preparation of tissue homogenates. Bovine tissues (from 59-month-old, 8-month-old and prion gene-deficient cows) were homogenized in lysis buffer [phosphate-buffered saline (PBS) containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 2 mM phenylmethylsulfonyl fluoride] using a bead shocker (FastPrep®; Thermo Electron) at 6.5 m/sec for 45 sec x 2 for the preparation of 10% (w/v) tissue homogenates. The hamster brain was homogenized in lysis buffer using an ultrasonic disintegrator (Sonifier 250; Branson), subjected to centrifugation at 5,000 x g for 5 min and stored at -80˚C until use.

Purification and horseradish peroxidase-labeling of mAbs. The characterization of the anti-PrP mAbs T2 and T17 is shown in Table I. Hybridomas producing T2 and T17 mAbs were grown separately in hybridoma-SFM (serum-free medium; Invitrogen) at 37˚C in a humidified 5% CO_2 incubator. Cell suspensions were subjected to centrifugation at 1,500 x g for 5 min before the supernatants were harvested. The mAbs were isolated from hybridoma supernatants using HiTrap Protein-G (Amersham Biosciences) and a PD-10 column (Amersham Biosciences) in accordance with the manufacturer's instructions. Harvested mAbs were labeled with horseradish peroxidase (HRP) using a Peroxidase-labeling Kit-SH (Dojindo) in accordance with the manufacturer's instructions.

SDS-PAGE. Protein samples were boiled in 2X SDS gel-loading buffer [90 mM Tris-HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue and 20% glycerol] for 5 min before being subjected to electrophoresis on SDS-12% polyacrylamide gel with molecular weight markers (Full-Range Rainbow™ Recombinant Protein Molecular Weight Marker RPN800; Amersham Biosciences). The gel was electro-blotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Biosciences) for 1 h at 120 mA. Resolved proteins on the membrane were visualized using Coomassie brilliant blue (CBB) staining.

Table I. Characterization of the anti-PrP mAbs.

mAb	Immune animal	Immunogen	Epitope
T2	Mouse	Recombinant mouse PrP (aa121-231)	132-156
T17	Mouse	Recombinant mouse PrP (aa121-231)	174-217

Western blotting. Electrophoresis of the 10% tissue homogenate was performed on SDS-12% polyacrylamide gel before electroblotting onto PVDF membranes using the method described above. The membranes were treated with Block Ace (Dainippon Sumitomo Pharmaceuticals) for 1 h at room temperature and subsequently incubated for 1 h at room temperature with T2 or HRP-labeled T2 (T2-HRP) antibodies (1 μg/ml in PBS with 0.1% Tween-20 or PBS-T). After three washes in PBS-T, the T2-incubated membranes were further incubated with a secondary antibody, peroxidase-labeled anti-mouse IgG (diluted 1:5000 in PBS-T) (Amersham Biosciences) for 1 h at room temperature. After three washes in PBS-T, the probed proteins were detected with a chemiluminescent substrate (ECL™ Detection Reagents; Amersham Biosciences) using a luminoimage analyzer (LAS-3000mini; Fujifilm).

Sandwich ELISA. Microtiter plates (Nunc-Immuno™ Modules; Nunc) were coated with 100 μl of purified capture mAbs (1 μg/ml) in 0.1 M carbonate buffer (pH 9.5) overnight at 4˚C and washed three times with PBS-T using a Nunc-Immuno™ Wash 8 (Nunc). The coated plates were then blocked with 200 μl of Block Ace (diluted 1:4 in PBS-T) for 1 h at room temperature, and subsequently rinsed three times with PBS-T. Diluted samples in PBS (100 μl) dispersed in 96-well plates were incubated for 1 h at room temperature. The plates were washed five times with PBS-T prior to treatment with 100 μl of HRP-labeled anti-PrP mAbs (0.5 μg/ml) and PBS-T in the wells. After washing with PBS-T, O-phenylenediamine (Sigma) solution was dispensed at 100 μl/well. After incubation for 30 min in the dark, each well was treated with 20 μl of H_2SO_4 (6 N), then the absorbance of each well was measured at 490 nm on a microplate reader (Bio-Rad).

Tissue preparation and immunohistochemistry. Each tissue sample (from a 6-month-old cow) was collected, fixed and embedded in resin (Technovit 8100; Heraeus Kulzer, Wehrheim, Germany) as described previously (24,25). Sections (4-μm) were sliced, mounted on silanized glass slides, subjected to immunohistochemistry, and observed using a fluorescence microscope (Olympus, Tokyo, Japan).

To observe distribution in the peripheral nervous system and cellular prion protein in the bovine ileum, sections were reacted with anti-PrP mAb T2 (1:100) or anti-PgP 9.5 pAb (1:100; Abcam, Tokyo, Japan) for 2 h at 37˚C, then further incubated with Alexa Fluor® 488-coupled goat anti-mouse IgG (H+L) antibody, or with goat anti-rabbit IgG (H+L) antibody

coupled with Alexa Fluor® 546 (5 μg/ml; Molecular Probes, Eugene, OR, USA) at room temperature for 30 min. Sections were then counterstained with DAPI.

Calculating the limit of detection for PrP levels. A limit of detection (LD) is defined as an analyte concentration that yields a signal intensity 3-fold the SD and mean intensity of the blank solution. According to Miller and Miller (26), the LD (y) is calculated based on the mean (y_B) and SD (S_B) of the signal from the blank control (vehicle signal) as shown in Equation (i). Therefore, the LD is defined if a sample is appropriate for PrP detection. The value of the calculated intercept on the ordinate is used as an estimate of y_B; i.e., the blank signal (26). To estimate S_B, statistical estimates of random errors on the ordinate [$S_{y/x}$; see Equation (ii)] were substituted for S_B (26). The $\hat{y}_i$-values are points on the calculated regression line corresponding to the individual x-values:

$$y = y_B + 3S_B \qquad \text{(i)}$$

$$S_{y/x} = \left\{ \frac{\sum_i (y_i - \hat{y}_i)^2}{n-2} \right\}^{\frac{1}{2}} \qquad \text{(ii)}$$

Results

The isolation and purification of the anti-PrP mAbs T2 and T17 from the supernatants were verified by SDS-PAGE. The bands of heavy- and light-chain mAbs were respectively visualized at 50 and 25 kDa by T2 and T17 antibodies with CBB staining. The purified mAbs were labeled with HRP using a peroxidase-labeling Kit-SH. The SH-reactive peroxidase in this kit has the maleimide to form a covalent bond with a sulfhydryl group of the reduced IgG form, thus binding 1 to 2 peroxidase molecules per IgG. PrP detection was performed by Western blotting to verify HRP-labeling before analysis with sELISA using the same mAbs. Using combinations of T2 and T7-HRP or T17 and T2-HRP, PrP was detected in a concentration-dependent fashion. However, PrP was not detected by T2-HRP or T17-HRP without any captured mAbs. This finding indicates that the novel sELISA method established in this study can detect PrP specifically. The combination of T2 as a captured mAb and T17-HRP as an identification mAb detected PrP with a higher sensitivity than T17 and T2-HRP combined. This is probably attributable to substantially more labeling of T17 than T2 with peroxidase molecules prior to sELISA.

PrP^C in different tissues was detected reliably using the sELISA method. The quantification test was calibrated using a linear standard curve detecting recombinant bovine PrP (Fig. 1). Based on Equation (i), using this assay, the LD value of bovine PrP was derived and recorded as 0.1651 ng (bovine PrP) per 100 μl. Of the tissue PrP contents observed using this assay (Fig. 2), the highest level was detected in the brain (13.3 μg/g tissue), followed by the longissimus capitis muscle (2.62 μg/g tissue; 5.2-fold less than the brain), iliocostalis thoracis muscle (2.53 μg/g tissue), splenius muscle (2.44 μg/g tissue), biceps femoris muscle (2.09 μg/g tissue), triceps brachii muscle (1.85 μg/g tissue), longissimus thoracis muscle (1.66 μg/g tissue), ileum (1.43 μg/g tissue), jejunum (1.41 μg/g tissue), duodenum (1.37 μg/g tissue), colon (1.33 μg/g tissue),

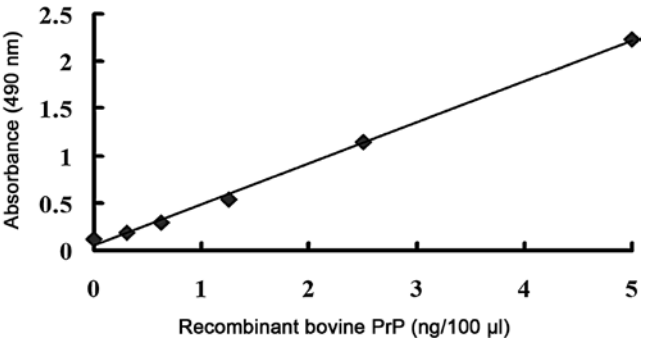

Figure 1. ELISA standard curve derived from the absorbance (490 nm) of recombinant bovine prion protein (PrP) in ng/100 μl. The ELISA standard curve was derived from recombinant bovine PrP, which was detected by the sandwich ELISA method with T2 and T17-HRP antibodies for quantitative determinations of PrP^C in bovine tissues. Each value is the mean of triplicate determinations.

cecum (1.20 μg/g tissue), apex linguae (1.12 μg/g tissue), omotransversarius muscle (0.91 μg/g tissue), posterior part of the corpus linguae (0.89 μg/g tissue), anterior part of the corpus linguae (0.83 μg/g tissue), radix linguae (0.75 μg/g tissue) and liver (0.25 μg/g tissue) (Fig. 2A). In the tongue, the PrP content was higher at the apex than at the root, while the ileum was found to manifest the highest PrP^C content in the intestines. Values for each tissue in the prion protein-deficient cows were quite low (Fig. 2B); these values were therefore judged to be background levels. A previous report indicated that Western blotting tests of the brain and spleen from prion protein-deficient cows were negative (23).

PgP 9.5-positive nerve cells and T2-positive PrP^C were detected in the plexuses of the jejunum (Fig. 3A, C and E) and ileum (Fig. 3B, D and F). The distributions of PrP^C were almost consistent with those of the nerve cells. PrP^C was expressed on the cell surface of nerve cells (Fig. 3B, D and F). sELISA indicated the presence of substantial amounts of neural tissue in most of the intestinal tissues (Fig. 2A).

Discussion

PrP^C expression is necessary for the development of TSEs (27,28). The chronological and spatial transport of PrP^{Sc} after oral infection remains unclear. After oral challenge, PrP^{Sc} is detected in frontline tissues such as the gastrointestinal tract and its associated lymphoreticular system in sheep, mice and hamsters; i.e., tissues that are highly susceptible to infection (29-35). PrP^C is the substrate for the formation of pathology-associated conformer PrP^{Sc} (36). However, studies on PrP^C distribution in cattle are limited (37-39). Although quantitative studies of PrP^C expression at the protein level have been documented in sheep, none have been attempted in cattle.

This study first labeled anti-PrP mAbs (purified from T2 and T17 hybridoma supernatants) with HRP before quantitative assessments with a novel sELISA method using these mAbs. PrP^C content in the peripheral tissues of cattle was then determined by the sELISA method. This sELISA system was able to detect PrP in both hamsters and cattle (data not shown). Moudjou *et al* reported that PrP^C content in sheep depends on the prion protein gene (*PRNP*) genotype (20). Moreover, according to Nakamura *et al*, certain polymorphisms of the

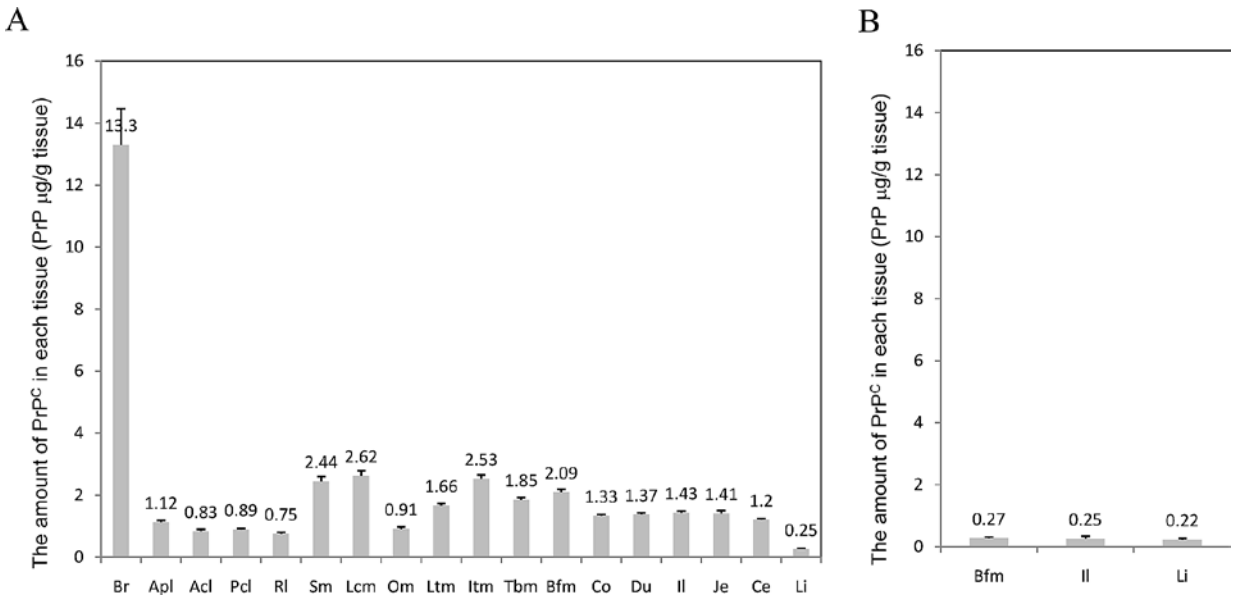

Figure 2. Quantitative determinations of the bovine cellular isoform of prion protein (PrP^C) in peripheral tissues/organs. The estimated content of the cellular isoform of prion protein (PrP^C) in bovine tissues (A) and prion protein-deficient cows (B) by the sandwich ELISA are expressed as μg protein/g of tissue. Tissues tested included the brain (Br), apex linguae (Apl), anterior part of the corpus linguae (Acl), posterior part of the corpus linguae (Pcl), radix linguae (Rl), splenius muscle (Sm), longissimus capitis muscle (Lcm), omotransversarius muscle (Om), longissimus thoracis muscle (Ltm), iliocostalis thoracis muscle (Itm), triceps brachii muscle (Tbm), biceps femoris muscle (Bfm), colon (Co), duodenum (Du), ileum (Il), jejunum (Je) cecum (Ce) and liver (Li). Each value represents the mean of triplicate determinations. Vertical error bars represent the standard deviations.

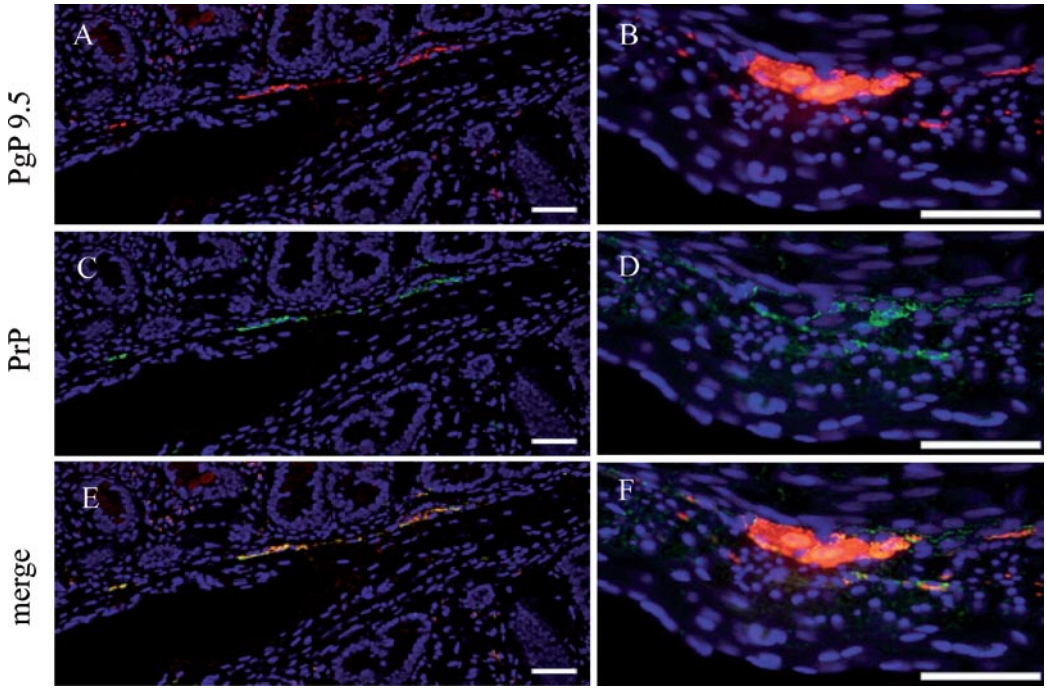

Figure 3. Immunohistochemical detection of the cellular isoform of prion protein (PrP^C) in plexuses of jejunum and ileum. (A and B) Substantial areas reacted with the PgP 9.5 antibody staining (red). (C and D) Substantial areas reacted with the T2 antibody staining (green). (E and F) Merged images of PgP 9.5 and T2 reactions. Bar, 50 μm.

bovine *PRNP* promoter region in Japanese Black cattle influence promoter activity (40). Therefore, it is necessary to study PrP^C content in the bovine tissues of various *PRNP* genotypes. The novel quantitative method used in the determination of the PrP^C content of various tissues in single cattle in this study facilitates an understanding of protein levels, which it was previously impractical to measure. This is the first study to reliably and quantitatively assay PrP^C contents in cattle.

An understanding of the distribution and expression level of PrP^C is important, as host-encoded PrP^C is required for the conversion to PrP^{Sc}. In addition, both the route and rate of PrP^{Sc} transport are controlled by the expression level of host-

encoded PrP^C (41,42). Using immunohistochemistry, we previously examined nerve distribution in the same tissues of the cattle used in the present study (43). The distribution of PrP^C is not always consistent with that of nerve cells. In conclusion, this study highlights the risk associated with bovine tongue, skeletal muscles and intestines as commercial products and the presence of PrP^C (a protein that has the potential to form PrP^{Sc}). To date, it has been impractical to quantitatively determine PrP^C levels. However, with the novel sELISA method employed in this study, PrP^C was detected in these tissues/organs. The presence of PrP^C in bovine ileum tissue was previously reported using immnunohistochemistry (44). However, the authors did not demonstrate the amount of PrP^C using sELISA or Western blotting. Another report by Iwata *et al* revealed PrP^{Sc} distribution in the ileum of BSE cases using Western blotting (45), but failed to detect PrP^C in bovine ileum tissues. Currently, sELISA and immunohistochemistry are the available methods used to evaluate PrP^C content in ileum tissue.

In the present study, it was additionally revealed that tissues from cattle lacking prion protein are suitable for use as a negative control for the risk analysis of prion disease. We also investigated the distribution of PrP^C in 2-week-old cows. The distribution pattern was found to be the same (data not shown). The availability of a qualitative and quantitative approach using the present novel sELISA method may facilitate the in-depth assessment of the risks of PrP^{Sc} accumulation in the peripheral tissues of cattle.

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