RESEARCH PAPER

Evaluation of rapid post-mortem test kits for bovine spongiform encephalopathy (BSE) screening in Japan: Their analytical sensitivity to atypical BSE prions

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ABSTRACT. A classical type of bovine spongiform encephalopathy (C-BSE), recognized in 1987, had a large impact on public health due to its zoonotic link to variant Creutzfeldt-Jakob disease by the human consumption of dietary products contaminated with the C-BSE prion. Thus, a number of countries implemented BSE surveillance using rapid post-mortem test kits that were approved for detection of the C-BSE prion in the cattle brain. However, as atypical BSE (L- and H-BSE) cases emerged in subsequent years, the efficacy of the kits for the detection of atypical BSE prions became a matter of concern. In response to this, laboratories in the European Union and Canada evaluated the kits used in their countries. Here, we carried out an evaluation study of NippiBL®, a kit currently used for BSE screening in Japan. By applying the kit to cattle brains of field cases of C-BSE and L-BSE, and an experimental case of H-BSE, we showed its comparable sensitivities to C, L-, and H-BSE prions, and satisfactory performance required by the European Food Safety Authority. In addition to NippiBL®, two kits (TeSeE® and FRELISA®) formerly used in Japan were effective for detection of the L-BSE prion, although the two kits were unable to be tested for the H-BSE prion due to the discontinuation of domestic sales during this study. These results indicate that BSE screening...
INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders that cause neuronal cell death and spongiosis in the brain of several mammalian species. In human beings, TSEs emerge in such forms as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome, fatal familial insomnia, and kuru. The causative agent is considered to be solely protein, referred to as 'prion', whose major constituents are disease-associated forms of prion protein (PrPSc, PrP refers to prion protein).1,2 PrPSc is a conformational isoform of glycosylphosphatidylinositol-anchored, non-pathogenic cellular prion protein (PrPC) encoded by the host gene, and it is partially resistant to proteolytic digestion by proteinase K (PK).2,3,4 A key event in prion propagation is the conversion of endogenous PrPSc to PrPSc, and PrPSc accumulates in the central nervous system of patients and animals. The cycles of conversion are triggered by pre-existing PrPSc as seeds, where the seeds are initially acquired by unknown processes, due to mutation of the PrPSc gene, or by the intake of external PrPSc. Accordingly, TSEs emerge as sporadic diseases of unspecified backgrounds, hereditary diseases, or infectious diseases.4

Bovine spongiform encephalopathy (BSE) is a TSE of cattle. A classical type of BSE (C-BSE) was first reported in 1987 in the United Kingdom,5 and its growing epidemic was recognized later in other countries by infection with the BSE prion through feeding contaminated meat-and-bone meal. Importantly, the epidemic of C-BSE posed an ensuing threat of zoonotic infection of humans after emergence of variant CJD cases in the 1990s, considered to be caused by the human consumption of beef products contaminated with the C-BSE prion.6,7 This prompted a number of countries to implement protective measures including BSE surveillance using rapid post-mortem test kits. These kits are based on the enzyme-linked immunosorbent assay (ELISA), Western blot, or immunochromatography, which detect PrPSc accumulated in the medulla oblongata at the level of the obex in cattle brains after proteolytic digestion and elimination of PrPC, or using antibodies that specifically recognize the conformation(s) of PrPSc. The performance of these kits in detection of the C-BSE prion was evaluated and approved by the European Commission and European Food Safety Authority (EFSA).8-10

Along with C-BSE, two novel atypical forms of BSE named H-type (H-BSE) and L-type BSE (L-BSE) were identified by the mid 2000s.11,12 Cases of L- and H-BSE are less common than those of C-BSE, but they have been reported in several countries of the European Union (EU) as well as Japan, Canada, and United States of America.11-25 Prions of atypical BSE are distinguished biochemically and pathologically from the C-BSE prion. The risk of transmission of atypical BSE prions to human beings is still under investigation. The L-BSE prion has been thought to be more virulent than the C-BSE prion in experimental transmission to non-human primates and transgenic mice expressing a human form of PrPSc 26-30 On the other hand, other studies showed inefficient transmission of the L-BSE prion to the transgenic mice, and inefficient in vitro conversion of a human form of PrPSc by the L-BSE prion31,32 So far, the H-BSE prion failed to be transmitted to the transgenic mice.29,31 Nevertheless, it is sensible to determine if the rapid tests in current use are also valid for the atypical BSE prions. From this point of view, evaluation studies were carried out on seven tests used in EU countries and three tests used in the Canadian national BSE surveillance program.33,34

In Japan, ELISA test kits such as ‘Platelia®’ and ‘TeSeE® BSE test kit’ (Bio-Rad Laboratories, Inc., Hercules, CA, USA), ‘FRELISA®'
BSE test kit’ (Fujirebio Inc., Tokyo, Japan), and ‘NippiBL® BSE test kit’ (Nippi Inc., Tokyo, Japan) were mainly used in the BSE screening, and NippiBL® is now the only available kit. In harmonization with reports from EU and Canadian laboratories as described above, we carried out an evaluation study of NippiBL®, together with TeSeE® and FRELISA®, to assess their competence to detect atypical BSE prions.

RESULTS

Performance of the Kits to Detect the C-BSE Prion

In a similar way to the preceding evaluation studies by EU and Canadian laboratories, the present study was designed to examine the performance of rapid ELISA tests to detect atypical L- and H-BSE prions in comparison with C-BSE prion. Hence, we began with reviews of the performance of three kits (NippiBL®, TeSeE®, and FRELISA®) using samples prepared from the brain of a C-BSE-affected cow, although the performances of these kits for detection of the C-BSE prion were already approved elsewhere. We also included the BetaPrion® BSE test kit (Analytik Jena AG - AJ Roboscreen GmbH, Leipzig, Germany) as a reference kit to compare our results with those of EFSA and EU laboratories.

The EFSA has defined two sensitivity criteria: the ‘diagnostic sensitivity’ is the ability to recognize confirmed positive test samples as positive, while the ‘analytical sensitivity’ is a detection limit of positive samples serially diluted by negative brain tissues (i.e., the dilution limit for detection). Consistent with the previous evaluation, all kits in the present study determined the brain samples positive for the C-BSE prion as positive. Thus, the kits fulfilled the criteria of diagnostic sensitivity.

Evaluation of the Kits for Atypical L- and H-BSE Prions

After reviewing the performances of the kits to detect the C-BSE prion, we then applied them to the brain tissues derived from an L-BSE affected cow (Fig. 2A to D). The results are summarized in Table 1. All kits distinguished the samples positive for the L-BSE prion and the samples of normal brains, without false-negative or false-positive signals (Table 1). Among the kits, BetaPrion® showed the best analytical sensitivity by reaching a detection limit at a 1: 64 dilution (2^6 dilution) of the brain of the L-BSE cow (Fig. 2D). Analytical sensitivities of NippiBL®, FRELISA®, and TeSeE® followed in the same descending order as determined for the C-BSE prion (Table 1). Next, we examined the performance of NippiBL® using the brain samples containing the H-BSE prion in a similar way, and obtained its detection limit at a 1: 16 dilution (2^4 dilution) of the brain (Fig. 2E, and Table 1). On four-parameter logistic model analysis, the dilution-response profiles of the kits for the brain samples of atypical L- and H-BSE cows were fitted to regression curves with adjusted R^2 values higher than 0.949 (Fig. 2A to E).
the stock homogenates, the homogenates were digested by PK and subjected to Western blot analysis for quantification of PrP Sc (Fig. 3A). Figure 3B shows correlations between the amounts of brain tissues and total signal intensities of PrP Sc, in which the total signal intensity of PrP Sc represents a sum of the intensities of the non-, mono-, and di-glycosylated forms of PrP Sc in Fig. 3A. The analysis showed that comparable signal intensities of PrP Sc were detected in the homogenates corresponding to 12.5 μg of brain tissue of the C-BSE cow, 100 to 200 μg of brain tissue of the L-BSE cow, and 40 to 50 μg of brain tissue of the H-BSE cow (Fig. 3A and B). Accordingly, the relative concentration of PrP Sc in the stock homogenates in the brains of C-, L-, and H-BSE cows were calculated to be approximately $2^1$, $1: 2^{1.5}$. In parallel, Table 1 indicates that all kits showed a detection limit for the C-BSE sample 2$^1$ to $2^{2}$-times higher than that for the L-BSE sample, and NippiBL® showed a detection limit for the H-BSE sample $2^{1}$-times higher than that for L-BSE. The parallelism in the relative concentration and detection limits of PrP Sc in the C-, L-, and H-BSE samples was an
indication of the invariable reactivity of each kit to the three types of BSE prions, though the overall sensitivities were different among the kits.

**Assessment of the Digestion Condition of NippiBL**

NippiBL employs a unique protocol of treating the brain tissues with a mixture of protease at 56°C for 10 min for digestion before applying them to the ELISA assay, whereas the other kits in the present study digest the tissues by PK at 37°C. Although NippiBL possessed adequate analytical sensitivity for the detection of atypical BSE prions (Fig. 2C and E, and Table 1), the line of evidence that PrPSc of atypical BSE prions was less resistant to proteolytic digestion under stringent conditions prompted us to examine if the digestion condition of NippiBL did not cause rapid or irregular decay of the signal intensities of PrPSc when applied to atypical BSE prions. To achieve this, we carried out a time-course study in which the brain samples of the C- and L-BSE cows were processed according to the protocol of NippiBL but with different duration of digestion for up to 20 min before applying the samples to the ELISA assay. As expected, the signal intensities obtained from the C-BSE brain samples remained at a stable level even when the samples were digested for 20 min (Fig. 4A). Under the condition, the L-BSE samples (high, mid, and low concentrations of PrPSc) showed a gradual and time-dependent decrease of the signal intensities during the digestion (Fig. 4A and B). However, the signals were sustained, and did not show a sudden fall or fluctuation that would potentially compromise the accuracy and reproducibility of the analysis. The narrow values of the standard error of the mean for the signal intensities of the triplicate L- and H-BSE samples in the dilution-response profiles (Fig. 2C and E) supported this observation.

**DISCUSSION**

In addition to the prior approval of three rapid post-mortem BSE test kits used in Japan for detection of the C-BSE prion, we examined the analytical performance of the kits for the detection of atypical BSE prions. Among the three kits, TeSeE and FRELISA were used...
FIGURE 2. Dilution-response profiles of the kits using the brain homogenates of L- and H-BSE cows. Raw data were plotted as the mean ± SEM from a set of triplicate wells. The dotted lines indicate thresholds of positivity defined by the manufacturers' protocols. Non-linear curve fitting was applied to the raw data using a four-parameter logistic model. (A – D) TeSeE<sup>®</sup>, FRELISA<sup>®</sup>, NippiBL<sup>®</sup>, and BetaPrion<sup>®</sup> tested on the samples prepared from the L-BSE cow, respectively. (E) NippiBL<sup>®</sup> tested on the samples prepared from the H-BSE cow.
until 2014, and NippiBL® has been used since 2006 and is currently the only available kit.

Our study showed that the kits correctly judged the positive samples as positive, and the negative samples prepared from normal brains as negative (Table 1). Although testing a large number of independent samples was beyond the scope of the present study, the results fulfilled the ‘diagnostic sensitivity’ and ‘specificity of the tests’ that the European Commission and the EFSA have defined as the ability to determine specimens of true positive animals to be positive, and true negative animals to be negative.8,39 With respect to the analytical sensitivity, the EFSA regulations require appropriate tests to be within a maximal 2 log10 inferiority range of the most sensitive test.39 In this regard, IDEXX HerdChek® BSE-scrapie (IDEXX Laboratories, Inc., Maine, USA) is viewed currently as the most sensitive test.9,33,34,38 Due to import regulations, we could not include IDEXX HerdChek® in the present study. Instead, based on the results of previous studies showing that TeSeE® (short protocol) and BetaPrion® satisfied the EFSA requirements for the detection of L- and H-BSE prions,33,34 we considered that FRELISA® and NippiBL®, whose sensitivities were between TeSeE® and BetaPrion®, met the EFSA requirements. In fact, when examined using the brain samples of the L-BSE cow, the detection limit of NippiBL® was only 2² factors lower than that of BetaPrion® (Fig. 2, and Table 1). Apart from the analytical sensitivity, it might be intriguing to consider how much tissue is

FIGURE 3. Western blot analysis after PK digestion to determine the relative amounts of PrPSc in the stock homogenates of the brain. (A) The stock homogenates of the brains of the C-, L-, and H-BSE cows were digested by PK, and aliquots of volumes of the digests corresponding to the indicated weights of tissues were subjected to Western blot analysis. PrPSc was detected using anti-PrP antibody 12F10, with the aid of a chemiluminescent detection reagent and a cooled CCD camera imaging system. The letters non-, mono-, and di- denote the non-, N-mono-, and N-di-glycosylated forms of PrPSc. (B) Signal intensities of the non-, mono-, and di-glycosylated forms of PrPSc in each lane in (A) were measured by ImageGuage software, combined as a total signal intensity of PrPSc, and plotted in relative magnitude by taking that of 50 µg of the C-BSE brain tissue as 10.0.
required for a single well of the ELISA plates since the amount differs depending on the kits. In BetaPrion®, for example, a well contains the PK-digested sample corresponding to 28 mg of brain tissue. Thus, the detection limit of BetaPrion® for the authentic C-BSE brain tissue at a 1:1,024 dilution suggests that BetaPrion® can detect PrPSc in as little as 27 µg of the brain of the C-BSE cow used in the present study (Table 1). In NippiBL®, a digested sample corresponding to 10 mg of brain tissue is applied to a well; thus, the detection limit of NippiBL® at a 1:256 dilution was equivalent to the ability to detect PrPSc in 40 µg of the brain of the C-BSE cow (Table 1). Importantly, despite the kits showing different overall analytical sensitivities, each kit showed no preferential reactivity to a particular type of BSE prions; TeSeE® and FRELISA® had comparable reactivities to C- and L-BSE prions, and NippiBL® had comparable reactivity to C-, L-, and H-BSE prions.

The medulla oblongata at the level of the obex is specified as a general specimen for rapid post-mortem BSE tests. The distribution and deposition of PrPSc vary in the regions of the brain. For example, we found by Western blot analysis that the cerebral specimens of the L-BSE cow we used in the present study contained an approximately 3-fold lower amount of PrPSc than the medulla oblongata at the level of the obex of this cow (data not shown). Similar to the preceding evaluation studies, the test specimens in the present study were not prepared from the medulla oblongata at the level of the obex but from other regions of the brains of the C-, L-, and H-BSE cows (see Materials and Methods). Despite the usage of specimens of the brain region not specified by the protocols, we think the results

FIGURE 4. Effect of the sample digestion condition of NippiBL® on detection of the L-BSE prion. (A) Brain samples of the C-BSE and L-BSE cows were digested according to the manufacturer’s protocol but for an extended time. For the L-BSE brain, samples of three different dilutions by normal brains were tested (a: 2−0.6 dilution, b: 2−1 dilution, c: 2−2 dilution). The arrowhead at the top of (A) indicates the digestion time set by the protocol (10 min). Data were plotted as the mean ± SEM from duplicate wells. The dotted lines indicate the thresholds of positivity defined by the protocol. The samples of normal brain (i.e., negative control) gave rise to negative signals throughout the assay. (B) Only data on the brain samples of the L-BSE cow in (A) were plotted for clarity.
of the present study strongly support the eligibility and efficacy of the test kits for the purpose of BSE screening.

In the present study, the samples for TeSeE®, FRELISA® and BetaPrion® were prepared by mixing the stock homogenates of the brains of the BSE affected cows in saline and the brain homogenate of normal cows in saline. The mixtures were centrifuged to discard the supernatant, and the pellet fractions of tissues (1-volume) were added with 2-volumes of the 1x concentrated homogenization buffers supplied by the kits (see Materials and Methods). This protocol provided an advantage in accurate dilutions of PrP Sc ranging from 2^{-0} to 2^{-11} in a specified volume of the pellet fractions, but it discarded soluble components of the tissues by centrifugation. Although we have not examined effects of the loss of soluble components on the performances of the kits, we conceived the ultimate goal of the present study was achieved because PrP Sc was expected to be retrieved in the pellet fractions almost quantitatively.43 Also, in comparison with the manufacturers’ protocols in which the concentrations of the homogenization buffers after addition to the tissues are at 0.80 ± 0.02x, the above protocol brought the concentrations of the buffers after addition to the pellet fractions to 0.67x. However, this did not seem to affect the performance of the test kits, since the buffers are 5% glucose (TeSeE®) or 50 mM Tris buffer supplemented with collagenase and DNase (FRELISA®). After addition of the homogenization buffers, the samples were homogenized and digested by PK according to the manufacturers’ protocols, so that the concentrations of PK and the other components such as urea and detergents were at the same as those indicated by the manufacturers. With regard to NippiBL®, the homogenization buffer contains Triton X-100 and urea, and the homogenization of the brain tissues is carried out in the buffer premixed with the proteases supplied with the kit. The manufacturer’s protocol instructs that the concentration of the homogenization buffer and the proteases is at 0.90 ± 0.02x after addition to tissues.35 In the present study, the concentrations of the buffer and the proteases after addition to the samples were as follows: 0.83x for the samples at a 2^{-1} dilution; 0.86x for the samples at a 2^{-2} dilution, 0.87x for the samples at a 2^{-3} dilution, 0.89x for the samples at 2^{-4} and 2^{-5} dilutions, and 0.90x for the samples at 2^{-6} to 2^{-11} dilutions.

An issue specific to NippiBL® is that it treats the brain tissues with a more aggressive digestion before applying them to the ELISA assay.35 We showed that the digestion condition of NippiBL® did not compromise the accuracy of detecting atypical BSE prions (Fig. 4). Conceivably, PrP Sc of atypical BSE prions is largely resistant to the digestion condition of NippiBL®, or PrP Sc might be degraded to some extent but the degrading peptides retain the epitopes of the antibodies for effective detection. With regard to NippiBL®, we incidentally found that the freezing and thawing of brain tissues in the NippiBL® homogenization buffer significantly impaired the resistance of PrP Sc to the proteases of the kit (data not shown), possibly due to the effect of components in the homogenization buffer such as Triton X-100 and urea.35 Of course, the manufacturer’s protocol does not instruct users to freeze brain specimens in the homogenization buffer prior to the digestion. This should be avoided.

In conclusion, the present study showed that NippiBL® is suitable for the detection of C-, L-, and H-BSE prions. Also, TeSeE® and FRELISA®, which were discontinued from use in Japan in 2014, were appropriate for the detection of C- and L-BSE prions. These results support the effectiveness of the current BSE surveillance program in Japan, and it is unlikely that cases of atypical BSE have been overlooked due to the test method being used.

**MATERIALS AND METHODS**

**Brain Tissues**

Brain tissues negative for the BSE prion (normal brain) were a pool of medulla oblongata tissues proximal to the level of the obex collected from four cows. These specimens were obtained from local abattoirs in Japan, and were determined to be negative for BSE by
Western blot and histopathological analyses at the National Institute of Infectious Diseases.

Brain tissues positive for the C-BSE prion were from the thalamus of a field case of a C-BSE cow identified in Ireland (case number: H02-551). The specimen was provided by Prof. W. Hall (University College of Dublin, Ireland) after permission for import given by the Ministry of Agriculture, Forestry and Fisheries, Japan.

Brain tissues positive for the L-BSE prion were from the cerebrum of a field case of a L-BSE cow identified in Japan. The cow (case number: JP24) was positive for BSE by routine screening using the Platelia BSE kit at a local meat-inspection laboratory, and determined as an L-BSE case by confirmatory analysis at the National Institute of Infectious Diseases. The DNA sequence of the PrP coding region of the cow had a synonymous codon of asparagine192 (AA) compared with that of Bos taurus PrP in a public database (accession number: AJ298878, AAC for asparagine).

Because no field case of H-BSE has been found to date in Japan, we utilized a cow experimentally infected with the H-BSE prion by intracranial administration (experimental code number: 9458). Tissues positive for the H-BSE prion were from the brain stem. The DNA sequence of the PrP coding region of this cow was identical to that of Bos taurus PrP in the database (accession number: AJ298878).

**Sample Preparation**

Stock homogenates of 40% (w/v) brains of C-BSE, L-BSE, and H-BSE cows were prepared in saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). Homogenization was carried out by vigorous shaking of the brains with ceramic YTZ® balls (2.7-mm diameter beads, Nikkato Co., Osaka, Japan) at 2,500 rpm for 5 min in a Multi-beads shocker® tissue disruptor (Yasui Kikai Co., Osaka, Japan). Stock homogenate of the normal brain was prepared in a similar way at a final concentration of 20% (w/v). For NippiBL®, aliquot weights of tissues of normal brains were mashed according to the manufacturer's protocol. Due to the scarcity of brain specimens of atypical BSE cows, the stock homogenates were serially diluted as described below.

**TeSeE**, **FRELISA**, and **BetaPrion**: The 40% stock brain homogenates of C-BSE and L-BSE cows were added to an equal volume of saline, then serially diluted to a 2 base logarithm up to $2^{-11}$ with 20% (w/v) normal brain homogenate to obtain 900 μL of 20% (w/v) brain homogenate (i.e., each sample contained 180 mg of brain tissues, in which the net amounts of the BSE-positive tissues were serially diluted). The samples were centrifuged at 19,000 xg at 4°C for 30 min, 600 μL of the supernatant was discarded, and the pellet fractions (equivalent to 180 mg of the brain per tube) were stored at −75°C until use. To obtain pellets of negative control tissues, aliquots of 900 μL of the 20% stock homogenate of the normal brain were centrifuged in the same way. Before the test, the pellet fractions were added to 600 μL of the homogenization buffer supplied by the kits to reconstitute 900 μL of 20% brain homogenate. The samples were then homogenized as instructed in the manufacturers’ protocols by using the ceramic beads supplied with the kits of TeSeE® and BetaPrion®, or by using ceramic YTZ® balls (1.5-mm diameter beads, Nikkato Co.) for FRELISA®. The homogenates were dispensed in triplicate tubes by the volumes indicated in the manufacturers’ protocols (i.e., dispense 250 μL per tube for TeSeE®, 250 μL for FRELISA®, and 200 μL for BetaPrion®) for digestion with PK, and the assay was performed according to the manufacturers’ protocols.

**NippiBL®**: The kit employs a unique protocol for sample preparation. The dissected brain tissues are mashed through mesh-bottomed cups (BioMasher), the mashed tissues are then directly added to 9-volumes of homogenization buffer supplemented with the proteases of the kit, and disrupted by ceramic beads to prepare 10% homogenate which is ready for digestion at 56°C. To adapt to the protocol, test samples were prepared in the following way to contain 70 mg of the total brain tissues but serially diluted amount of the BSE-positive tissues: a sample at a $2^{-1}$ dilution was prepared by the
addition of mashed normal brain (35 mg) with 88 μL of the 40% homogenates of BSE-positive brains (equivalent to 35 mg tissue), a sample at a 2⁻² dilution was prepared by the addition of mashed normal brain (52 mg) with 44 μL of the 40% homogenates of BSE-positive brains (equivalent to 18 mg tissue), and samples at 2⁻³ to 2⁻¹¹ dilutions were prepared by the addition of 70 ± 5.6 mg (mean ± SD) of mashed normal brain with appropriate volumes of the 40% homogenates of BSE-positive brains. Pieces of 70 mg of the normal brains were mashed and used as negative controls. The samples were added to 9-volumes of the homogenizing buffer containing the proteases to adjust the tissue concentration to 10%. Then, as instructed by the manufacturer’s protocol, the samples were homogenized by using the ceramic beads that were supplied with the kit, and subjected immediately to digestion. After digestion, the samples were dispensed to the wells of the kit in triplicate, and the assay was performed according to the manufacturer’s protocol.

**Execution of the Test**

In the present study, we examined one rapid test kit for either of the brain samples of C-, L-, or H-BSE cow per day, and did not carry out simultaneous examination of different kits or different brain samples on the same day. To minimize variability, only two operators participated in the assay. A Model 680 microplate reader (Bio-Rad Laboratories, Inc.) and an ARVO X4 microplate reader (PerkinElmer Inc., Waltham, MA, USA) were used to measure the absorbance indicated by the manufacturers’ protocols (450 nm for reading; 620 nm for reference). All procedures were carried out according to the biosafety guidelines of the National Institute of Infectious Diseases, and the National Institute of Animal Health.

**Data Analysis**

True-positive and pseudo-positive thresholds were defined by the manufacturers’ protocols. If more than two wells in the triplicate wells were positive or pseudo-positive, the overall result was judged as positive. The detection limit was defined as the maximum dilution factor where the overall result was positive. Fitting analysis by a four-parameter logistic model was carried out using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) with the constraint of the maximum absorbance being less than 3.5. Adjusted R-squared (adjusted $R^2$) values were calculated by Prism 6 software using the following equation: $\text{Adjusted } R^2 = 1 - \frac{\text{SS}_{\text{residuals}}/(n-K)}{\text{SS}_{\text{total}}/(n-1)}$, where SS$_{\text{residuals}}$ is the sum of squares of the difference of each point from the fitted-curve, SS$_{\text{total}}$ is the square of the difference of the points from the mean of all absorbance, n is the number of data points, and K is the number of parameters fitted by the regression analysis.

**Western Blot Analysis**

The stock homogenates of the C-, L-, and H-BSE brains were diluted to 20% (w/v) with saline, and then 50 μL of the 20% (w/v) homogenates (equivalent to 10 mg of tissues) were added with an equal volume of a buffer consisting of 4% zwittergent® 3–14 (Merck Millipore, Darmstadt, Germany), 1% lauroylsarcosine sodium salt (Sigma-Aldrich, St. Louis, MO, USA), 100 mM NaCl, 50 mM Tris-HCl (pH 7.5). The samples were added to 0.625 μL of 80 mg/mL collagenase (Wako Pure Chemical Industries, Osaka, Japan), and incubated at 37°C for 30 min. After brief sonication, the samples were added to 1 μL of PK (at a final concentration of 50 μg/mL; Roche Diagnostics, Basel, Switzerland), and incubated at 37°C for 30 min. The digestion was stopped by the addition of 4-(2-aminoethyl)benzenesulfonyl fluoride at a final concentration of 2 mM (Roche Diagnostics). Following the addition of 50 μL of a mixture of 2-butanol and methanol (5/1, v/v), the samples were centrifuged at 18,000 xg for 10 min at 23°C. The pellet was dissolved in lithium dodecyl sulfate sample buffer (Thermo Fisher Scientific Inc., Novex™, Carlsbad, CA, USA) supplemented with 80 mM dithiothreitol, heated at 100°C for...
5 min, and aliquots of the samples were subjected to gel electrophoresis using a NuPAGE® Novex™ 12% Bis-Tris gel (Thermo Fisher Scientific Inc., Invitrogen™) and NuPAGE® MOPS-sodium dodecyl sulfate running buffer (Thermo Fisher Scientific Inc., Novex™). After electrophoresis, proteins were transferred to an Immobilon-P PVDF membrane (Merck Millipore) at 220 mA for 60 min using Tris-glycine buffer (Bio-Rad Laboratories, Inc.) supplemented with 20% methanol. The membrane was incubated at 4°C overnight with anti-prion protein 12F10 antibody (epitope: G153SDYEDRYRENMHRYPNQ171 of bovine PrP; Cayman Chemical, Ann Arbor, MI, USA) at 0.16 μg/mL in Can Get Signal®-1 immunoreaction enhancer solution (Toyobo Co., Ltd., Osaka, Japan). After washing the membrane with 0.05% Tween 20 in phosphate-buffered saline, the membrane was incubated at room temperature for 2 h with horseradish peroxidase-conjugated AffiniPure F(ab’)2 anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., PA, USA) at 0.1 μg/mL in Can Get Signal®-2 solution (Toyobo Co., Ltd.). Detection was carried out using SuperSignal™ West Dura Extended Duration Substrate (Thermo Fisher Scientific Inc., Thermo Scientific™) and a FluorChem IS-8044 imaging system (ProteinSimple, San Jose, CA, USA). Captured images were stored as TIFF files, and signal intensities were quantified by ImageGauge software (Fuji Photo Film, Tokyo, Japan).

**Examination of the Effects of the Digestion Condition of NippiBL®**

To examine the effects of the digestion condition of NippiBL® on stability of signal intensities of atypical BSE prions, a sample of the C-BSE cattle brain diluted to 2\(^{-5}\) by mashed negative brains, and three samples of the L-BSE cattle brain diluted to 2\(^{-0.6}\) (i.e., 1.5-fold), 2\(^{-1}\), and 2\(^{-2}\) by mashed negative brains were prepared using the method described above. These dilutions were chosen with the expectation of signal intensities (i.e., absorbance at 450 nm) between 1.0 and 3.0, based on the data of the dilution-response profiles of NippiBL® shown in Fig. 2. The samples were processed according to the protocol of NippiBL®, but by setting the digestion time at 5, 10, 15, and 20 min. Normal brain tissue was processed in the same way. After digestion, the samples were dispensed to the duplicate wells of the kit, and developed for detection according to the manufacturer’s protocol.

**ABBREVIATIONS**

- BSE bovine spongiform encephalopathy
- CJD Creutzfeldt-Jakob disease
- EFSA European Food Safety Authority
- ELISA enzyme-linked immunosorbent assay
- PK proteinase K
- PrP\(_C\) cellular prion protein
- PrP\(_{Sc}\) disease-associated forms of prion protein
- TSE transmissible spongiform encephalopathy

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

**ACKNOWLEDGMENTS**

The authors thank Dr. Tetsutaro Sata (former affiliation; National Institute of Infectious Diseases, Tokyo, Japan) and Prof. William. Hall (University College of Dublin, Dublin, Ireland) for providing us with specimens of the C-BSE cow, Drs. Hiroyuki Okada, Morikazu Imamura, Yuichi Matsuura, Kentaro Masujin, and Kohitaro Miyazawa (National Institute of Animal Health, Ibaraki, Japan) for specimens of the H-BSE cow. The specimens of the L-BSE cow were given to us by courtesy of the abattoir and Meat Inspection Center of Sasebo city (Nagasaki, Japan). We thank Dr. Kentaro Hanada (National Institute of Infectious Diseases, Tokyo, Japan) for advice regarding this study.

**FUNDING**

This study was supported by the Ministry of Health, Labor and Welfare, Japan under Grant H26-Shokuhin-Ippan-004. The funder did not...
participate in the study design, data collection and analysis, or preparation of the manuscript.

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