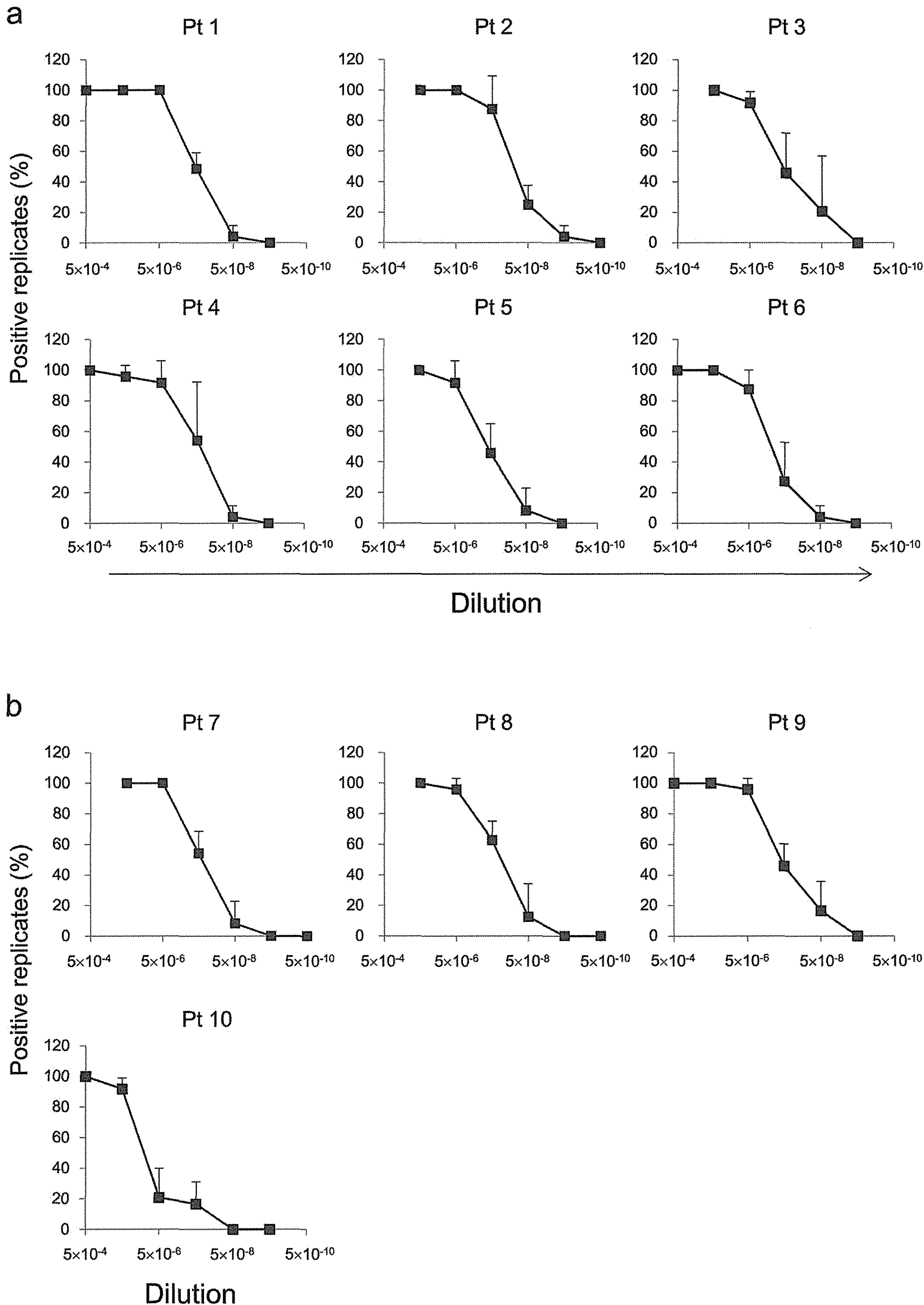

Fig 2. End-point RT-QUIC analysis of 10 brain specimens from patients with prion diseases. End-point RT-QUIC assay was performed three times. (a) Brain tissues from six patients with sCJD-MM1 were used to seed the RT-QUIC reaction. (b) Samples of GSS-P102L, sCJD-MV2, and sCJD-MM2 (cortical and thalamic forms) were used to seed RT-QUIC reaction.

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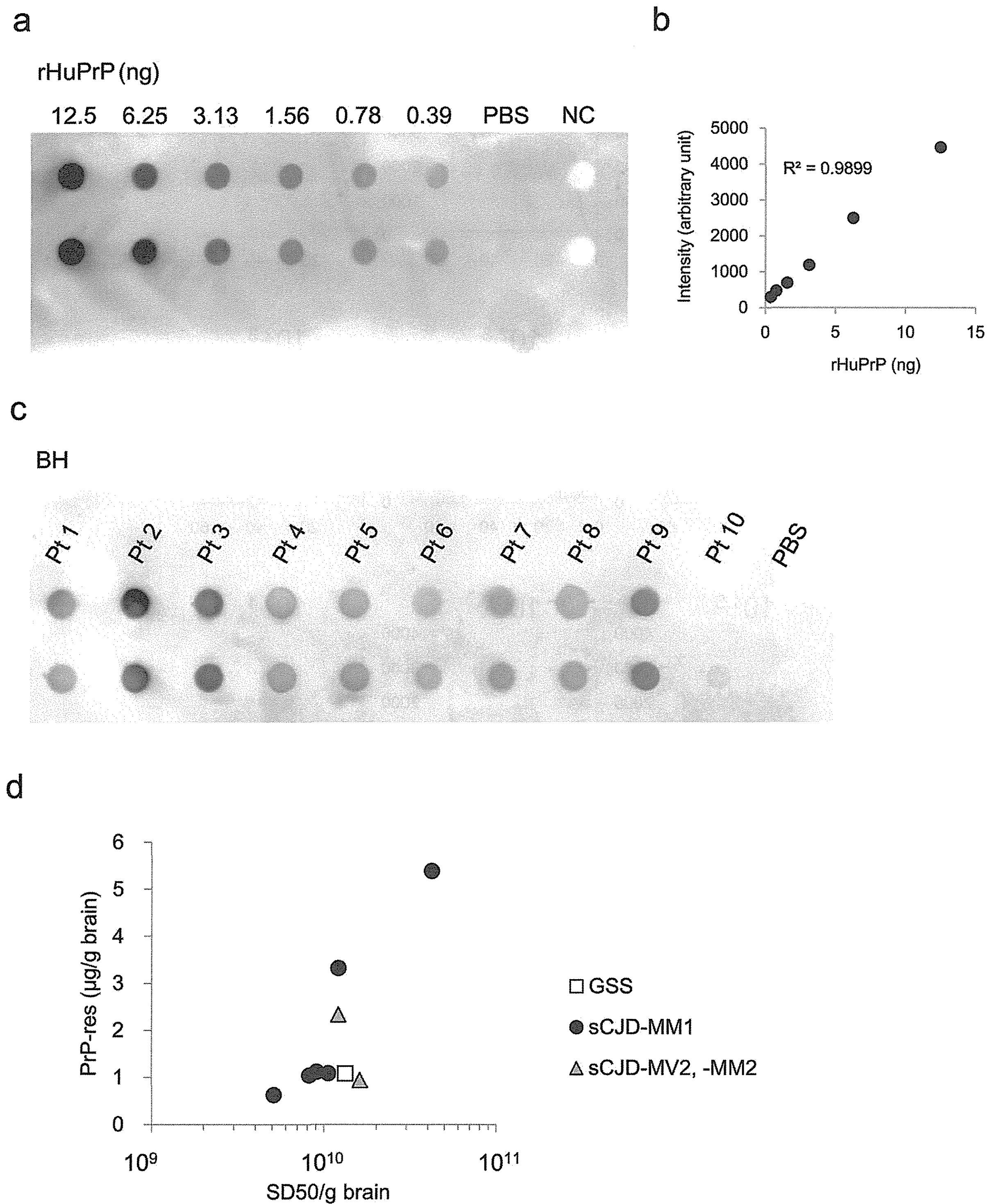

Fig 3. Correlation between SD_{50} and PrP-res in the brain. (a) Human recPrP was serial diluted and tested by dot blotting. (b) A standard curve was constructed using diluted human recPrP. (c) Dot-blotting of BHs from patients with prion diseases. Pt 10 (MM2-thalamic form) had a very weak signal and fell below the limit of detection. (d) There was a linear correlation between SD_{50} and the level of PrP^{Sc} in nine patient's brains ($y = 1.281 \times 10^{-10}x$, $R^2 = 0.7192$). NC = Normal brain homogenate. y = value of PrP-res (µg/g brain). x = value of SD_{50}/g brain

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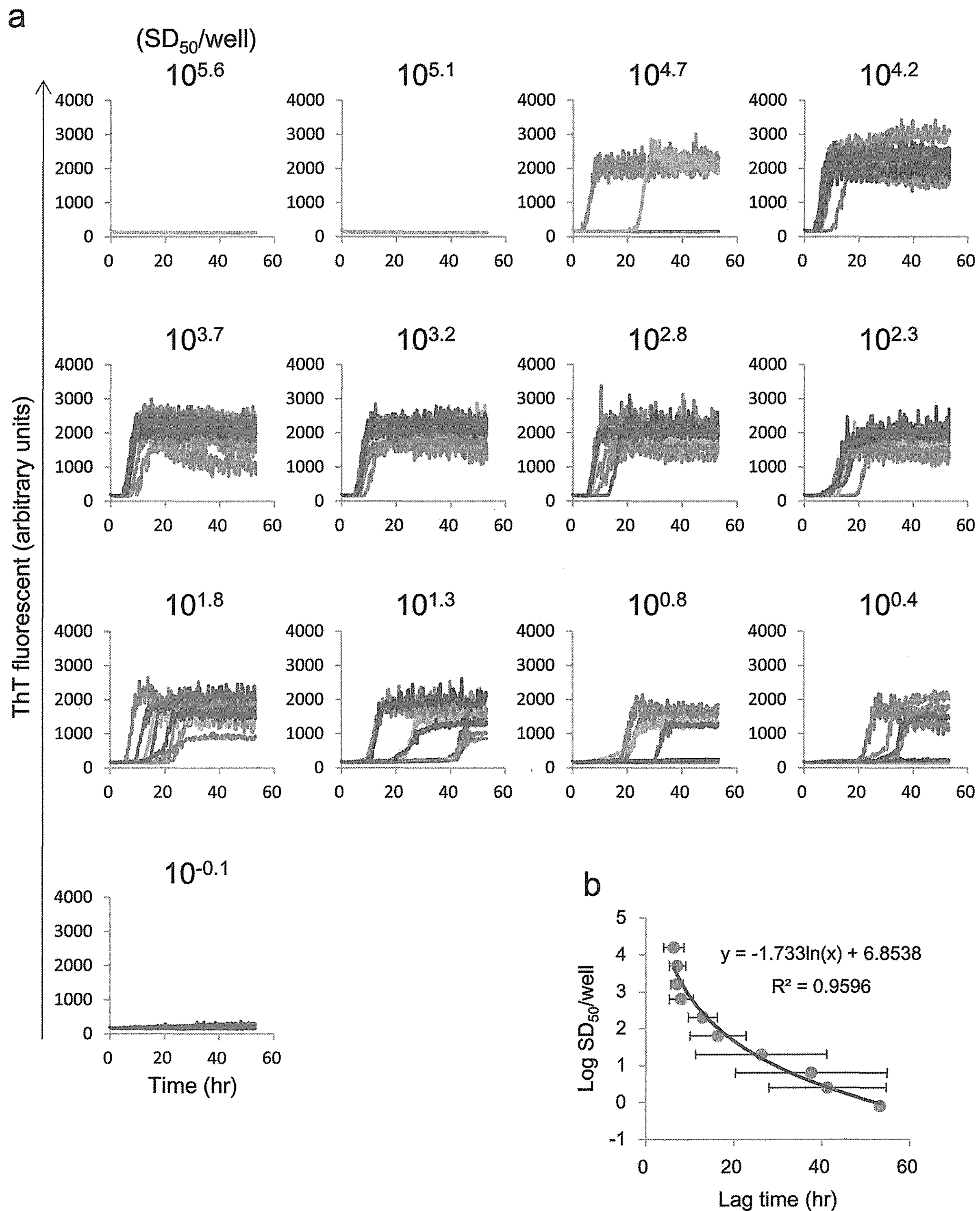

Fig 4. Preparation of standard curves based on lag phase and aggregate area in RT-QUIC. (a) Brain specimen from a patient with sCJD (patient 4) was subjected to serial three-fold dilution and RT-QUIC reaction, with four replicates for each dilution. (b) Standard curves (gray line) based on lag phase.

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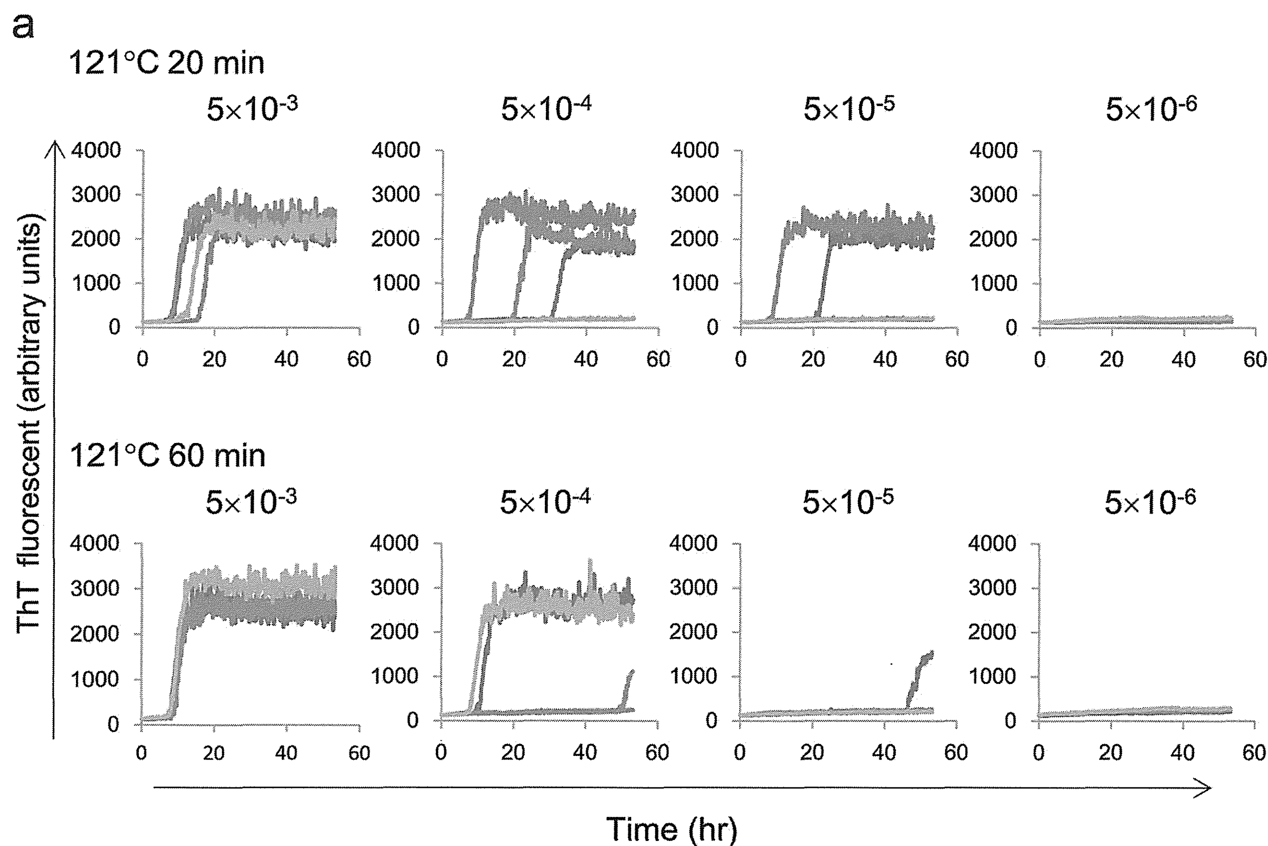
a



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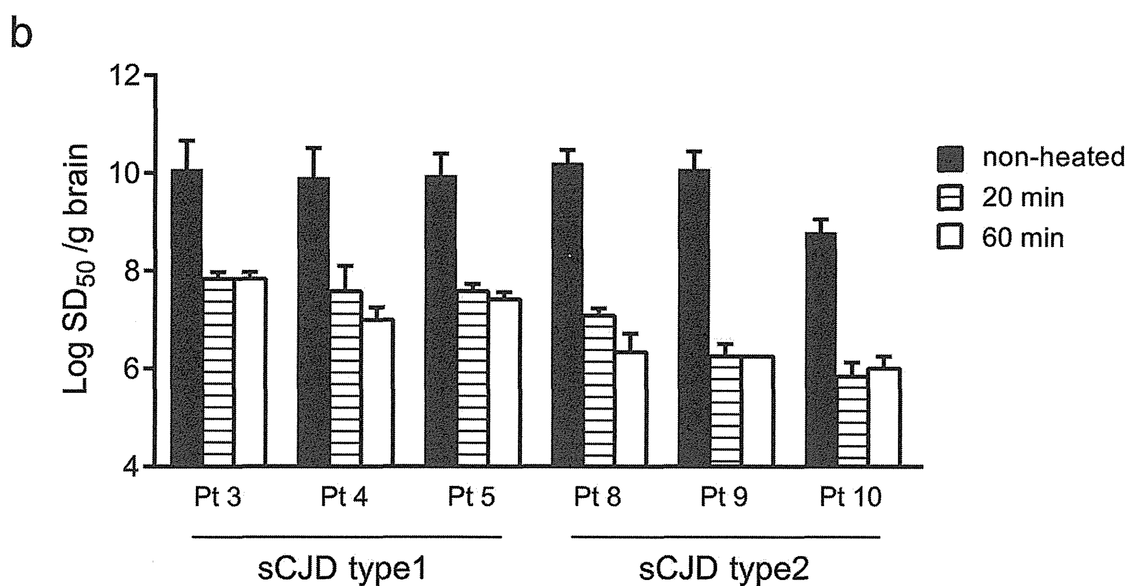

Fig 5. Reduction of seeding activity by heat treatment. (a) Brain from patient with sCJD-MM1 (patient 3) was treated at 121°C for 20 min or 60 min, and seeding activity was tested by end-point RT-QUIC. (b) Remaining SD_{50} after heat treatment. Black represents non-treated CJD-BH (patients 3–5 and 8–10). Horizontal stripes and white represents SD_{50} after heat treatment for 20 min and 60 min, respectively. Heat treatment caused reduction of SD_{50} (2.25 to 3.88 orders of magnitude). Data are presented as means ± standard deviation.

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121°C for 20 min and 60 min yielded an SD_{50} value of $10^{7.75}$ and $10^{7.5}$/g brain. CJD type 2 (patients 8–10) tended to be affected by heat treatment more than CJD type 1 (patients 3–5).

Discussion

The end-point RT-QUIC assay enables us to quantitate human prion seeding activity in brains from patients with prion disease. In brains from sCJD-MM1, the average SD_{50} was $10^{10.06}$. According to a previous report, LD_{50} of brain tissues from patients with sCJD-MM1 falls within the range 10^{7-9} LD_{50} /g [18]. Although our bioassays of these brain tissues is ongoing, it is likely that SD_{50} could be 10–100 times more sensitive than LD_{50}, because similar differences between SD_{50} and LD_{50} were seen in experiments using hamster prion 263K [23]. Notably, it was also possible to determine SD_{50} using the MM2-cortical form, the MM2-thalamic form, MV2, and a case of GSS-P102L.

There was a linear correlation between SD_{50} and the level of PrP-res in the brains of six patients with CJD-MM1 ($R^2 = 0.8173$). Based on estimation by dot-blot analysis, 1 SD_{50} was equivalent to 0.1 fg of PrP-res, suggesting that our RT-QUIC can detect PrP over a wider range than conventional Western blotting or ELISA. SD_{50} from all samples (10 patients, including MM2-cortical, MM2-thalamic, MV2, and GSS-P102L) exhibited a low correlation with the level of PrP-res ($R^2 = 0.7532$), possibly because resistance to protease digestion of PrP is not always the same as seeding activity.

Inhibition of the RT-QUIC reaction were seen in samples seeded with 1 to 0.1% brain tissue and 0.2% (5×10^{-2} dilution) spleen tissue (S2 Fig). A spleen specimens from a patient with sCJD yielded an SD_{50} value of $10^{7.5}$/g tissue. There was no positive reaction when normal spleen tissue was used as the seed (data not shown). Tissue samples include an inhibitor of RT-QUIC reaction; therefore, in order to quantitate seeding activity in tissue samples, it is important to reduce the concentration of this inhibitor by dilution.

Effective decontamination methods are essential in order to avoid iatrogenic transmission of prion diseases by contaminated medical equipment [27, 28]. PrP^{Sc} is resistant to chemical disinfectants such as ethanol and formaldehyde. By contrast, bioassays revealed that autoclaving at 121°C for 30 min and 60 min reduced infectivity of CJD inoculated mouse brain by 3.1 and 3.8 log_{10} units/g tissue, respectively [29]. Here, we conducted our preliminary assessments using human brain treated with simple heating. Heat treatments at 121°C for 20 min and 60 min reduced SD_{50} by 2.25 and 3.88 orders of magnitude, respectively. In the future, we will have to reassess LD_{50} using humanized mice and evaluate SD_{50} by RT-QUIC in all organs. Because RT-QUIC is an easy and rapid assay for determining prion activity, this approach provides a new way to evaluate biological patient specimens and reassess the safety of donated organs.

Supporting Information

S1 Fig. Non-prion brain has no seeding activity. Brain specimens from patients with prion disease (Patients 2–10) and non-prion disease were diluted (5×10^{-5}) and subjected to RT-QUIC reaction. Positive reactions were observed in RT-QUIC reactions using brain tissues from patients with prion disease. There was no response in the presence of non-prion samples (Non-PrD 1 and 2).
(PDF)

S2 Fig. Seeding activity was detected in spleen tissue from patient with sCJD. Spleen specimen from the patient with sCJD was diluted (5×10^{-2} to 5×10^{-5}) and subjected to RT-QUIC reaction.
(PDF)

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Author Contributions

Conceived and designed the experiments: HT K. Sano K. Satoh RA NN. Performed the experiments: HT TF TN TM. Analyzed the data: HT K. Satoh DI RA NN. Contributed reagents/materials/analysis tools: BM MT YI MY. Wrote the paper: HT K. Satoh RA NN.

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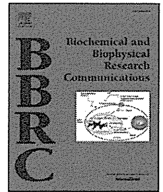

L-Arginine ethylester enhances *in vitro* amplification of PrP^{Sc} in macaques with atypical L-type bovine spongiform encephalopathy and enables presymptomatic detection of PrP^{Sc} in the bodily fluids

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ABSTRACT

Protease-resistant, misfolded isoforms (PrP^{Sc}) of a normal cellular prion protein (PrP^{C}) in the bodily fluids, including blood, urine, and saliva, are expected to be useful diagnostic markers of prion diseases, and nonhuman primate models are suited for performing valid diagnostic tests for human Creutzfeldt-Jakob disease (CJD). We developed an effective amplification method for PrP^{Sc} derived from macaques infected with the atypical L-type bovine spongiform encephalopathy (L-BSE) prion by using mouse brain homogenate as a substrate in the presence of polyanions and L-arginine ethylester. This method was highly sensitive and detected PrP^{Sc} in infected brain homogenate diluted up to 10^{10} by sequential amplification. This method in combination with PrP^{Sc} precipitation by sodium phosphotungstic acid is capable of amplifying very small amounts of PrP^{Sc} contained in the cerebrospinal fluid (CSF), saliva, urine, and plasma of macaques that have been intracerebrally inoculated with the L-BSE prion. Furthermore, PrP^{Sc} was detectable in the saliva or urine samples as well as CSF samples obtained at the preclinical phases of the disease. Thus, our novel method may be useful for furthering the understanding of bodily fluid leakage of PrP^{Sc} in nonhuman primate models.

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1. Introduction

Prion diseases are characterized by the pronounced accumulation of the misfolded isoforms (PrP^{Sc}) of a normal cellular protein (PrP^{C}) in the central nervous system [1,2]. PrP^{Sc} exhibit several peculiar pathophysiological characteristics: They are rich in beta-sheet structures [3,4], resistant to protease digestion and various inactivating treatments [5], and considered to be the infectious agents of fatal neurodegenerative diseases in both humans and animals [6].

Bovine spongiform encephalopathy (BSE) is an emerging prion disease that first appeared in the United Kingdom [7]. Since variant Creutzfeldt-Jakob disease (vCJD), a human neurodegenerative disease, is suspected to be attributable to infectious agents associated with BSE [8–10], infected cattle should be identified and eradicated as part of preventive health management. Several etiological studies of BSE prion-infected cattle have identified BSE prion types distinct from that of the classical BSE (C-BSE) in many countries, although these are rare. These atypical BSEs have been classified as H- or L-types according to the molecular weight of the non-glycosylated band derived from the protease-resistant PrP^{Sc} core [11]. These new types of BSE prions are transmissible to transgenic mice expressing human prion protein [12,13] and to nonhuman primates [14], and infected animals develop the diseases after a shorter incubation period than that observed for animals with C-BSE. In addition, L-BSE prion was transmissible to nonhuman primates by oral administration [15]. Therefore, identification of L-BSE-affected animals is attracting considerable attention of for not only animal hygiene management, but also from the human public health perspective.

Abbreviations: PrP^{Sc}, Pathogenic form of prion protein; PrP^{C}, cellular prion protein; CJD, Creutzfeldt–Jakob disease; BSE, Bovine spongiform encephalopathy; CSF, cerebrospinal fluid; PMCA, protein misfolding cyclic amplification; LAE, L-arginine ethylester; NaPTA, sodium phosphotungstic acid; Poly-A, polyadenylic acid potassium salt; PPS, sodium polyphosphate; PK, proteinase K; WB, western blotting.

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Nonhuman primate models are well suited for validating diagnostics for human prion diseases, because C-BSE in macaques resembled vCJD in humans in many aspects, including the pathological features of the brain tissue, biochemical characteristics of the PrP^{Sc} glycoform profile, and PrP^{Sc} distribution in the peripheral tissues [16]. In order to effectively prevent the spread of prion diseases, it is necessary to detect PrP^{Sc} as soon after infection as possible. It is now possible to amplify PrP^{Sc} *in vitro* using the protein misfolding cyclic amplification (PMCA) technique [17]. PMCA has been applied to the detection of bovine C-BSE PrP^{Sc} in cattle [18] and macaques [19]. In our previous study, we demonstrated the PrP^{Sc} was detectable in the bodily fluids such as cerebrospinal fluid (CSF) and blood as well as in various tissues of C-BSE prion-infected macaques. However, the PMCA method developed for macaque C-BSE PrP^{Sc} was not effective for amplification of macaque L-BSE PrP^{Sc}.

In this study, we developed a highly efficient PMCA method suitable for amplification of cynomolgus macaque L-BSE PrP^{Sc}. We further investigated PrP^{Sc} levels in the bodily fluids during the period from the latent to terminal stages of the disease.

2. Materials and methods

2.1. L-BSE prion-infected macaques

The study on nonhuman primates was conducted according to the Rules for Animal Care and Management of the Tsukuba Primate Research Center [20] and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [21]. The cynomolgus macaques (*Macaca fascicularis*) used in this study originated from Malaysia, and were bred at the Tsukuba Primate Research Center of the National Institutes of Biomedical Innovation, Health and Nutrition. Transmission experiments were approved by the Animal Welfare and Animal Care and Use Committee (approval ID: DS23-41) and Animal Ethics Biosafety Committee (approval ID: BSL3-R-10.04, BSL3-R-11.09, and BSL3-R-12.07) of the National Institutes of Biomedical Innovation, Health and Nutrition. The brain homogenate (200 μl of a 10% brain homogenate) derived from an L-BSE prion-infected macaque (#15) [22] was intracerebrally administered to two male macaques (#22 and #23) that were 1.4 years of age. These macaques were homozygous for codon 129 methionine/methionine (M/M) and 219 glutamic acid/glutamic acid (E/E). The second passage macaques were housed in biosafety level three animal rooms, and their clinical status was monitored daily. After 23–24 months, the animals were euthanized by anesthesia overdose following evidence of progressive neurologic dysfunction such as tremor and paralysis. Two healthy macaques were used as uninfected controls in the PMCA assay of bodily fluids.

2.2. Preparation of bodily fluid samples

The CSF, saliva, urine, and blood samples were collected from two macaques under anesthesia at intervals of approximately 3–7.5 months after inoculation. The bodily fluids, except saliva, were also collected 14–15 days before inoculation. The heparinized blood samples were centrifuged at 1500 *g* for 15 min, and the plasma fraction was recovered. These bodily fluids were stored in small aliquots at −80 °C. Before use in PMCA analysis, each sample was concentrated by precipitation with sodium phosphotungstic acid (NaPTA) [23]. Briefly, the samples were thawed and then centrifuged at 600 *g* for 1 min to remove large aggregates and cell debris, and the supernatants were used for precipitation. The supernatants (1000 μl from urine and plasma samples; 300–600 μl from CSF samples, and 400–1000 μl from saliva samples) were mixed with 4% NaPTA-170 mM $MgCl_2$-10 mM Tris (hydroxymethyl) aminomethane (pH7.5) in a 15:1 ratio. The mixtures were incubated at 37 °C for 18 h with continuous agitation. The samples were then centrifuged at 20000 *g* for 30 min at 25 °C. The supernatants were completely removed, and the precipitates were stored at −80 °C until analysis.

2.3. Preparation of PrP^{C} substrates

In our previous study, we found that cynomolgus macaque C-BSE PrP^{Sc} effectively converted mouse PrP^{C} to a proteinase K (PK)-resistant form [19]. Therefore, we used mouse brain homogenates as PrP^{C} substrate in the present study. To avoid contamination, normal brain homogenates were prepared in a laboratory in which infected materials had never been handled. Briefly, brains of wild-type mice (ICR) were homogenized in the presence of a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at a 10% (w/v) concentration in PBS containing 1% Triton X-100 and 4 mM EDTA. The homogenates were then centrifuged at 4500 *g* for 5 min, and the supernatant was used as the PrP^{C} substrate as previously described [19]. Heparin sodium salt (Santa Cruz Biotechnology, Texas), polyadenylic acid potassium salt (Poly-A, Sigma–Aldrich, Missouri), and sodium polyphosphate (PPS, Sigma–Aldrich) were dissolved in PBS and added to the PrP^{C} substrate at final concentrations of 100 μg/ml, 100 μg/ml, and 0.05%, respectively. Optimal concentration of each polyanion was determined in our preliminary experiments.

For efficient amplification of L-BSE PrP^{Sc}, L-arginine ethylester dihydrochloride (LAE, Sigma–Aldrich) was used as an additive to promote PMCA reaction. LAE was dissolved in distilled water at 2 M, and pH was adjusted to 7.0 by adding 1 N sodium hydroxide aqueous solution. To estimate the optimal concentration of LAE, the solution (final concentrations of 0–100 mM LAE) was added to the PrP^{C} substrate containing the polyanion cocktail.

2.4. PMCA and western blotting

For the amplification of brain PrP^{Sc}, the L-BSE prion-infected brain homogenate of macaque #14 [22] was serially diluted from 10^{-3} to 10^{-11} with the mouse PrP^{C} substrate in an electron beam-irradiated 8-strip polystyrene tube (total volume, 80 μl) [18]. Amplification was carried out with an Elestein 070-CPR (Elekon Science Corporation, Chiba, Japan). Serial PMCA was performed in quadruplicate using the four-step amplification program with 40 cycles of sonication in which a 15-s oscillation and subsequent incubations at 31 °C for 1 h were repeated 10 times; 15-s oscillation and subsequent incubations at 33 °C for 1 h were repeated 10 times; an intermittent oscillation (3-s pulse oscillation was repeated five times at 0.1-s intervals) and subsequent incubations at 35 °C for 1 h were repeated 10 times; and intermittent oscillations (3-s pulse oscillation was repeated five times at 0.1-s intervals) and subsequent incubation at 37 °C for 1 h were repeated 10 times. The amplified product obtained after the first round of amplification was diluted 1:5 with the PrP^{C} substrate, and a second round of amplification was performed. This process was repeated for a maximum of seven times. After each round of amplification, the amplified products were digested with proteinase K (PK, 100 μg/ml) and incubated at 37 °C for 1 h. The digested materials were separated by SDS-PAGE and analyzed by western blotting (WB) as previously described [24].

For amplifying PrP^{Sc} in bodily fluids from L-BSE prion-inoculated macaques, the frozen precipitates were dissolved in 20 μl of PBS and used as a seed. The concentration rates were 50 in the urine and plasma samples, 15–30 in the CSF samples, and 20–50 in the saliva samples. The mouse PrP^{C} substrate containing polyanion cocktail (100 μg/ml heparin, 100 μg/ml poly-A and 0.05% PPS) and

12.5 mM of LAE was mixed with a 1/10 volume (8 μl) of concentrated bodily fluids in 8-strip polystyrene tubes (total volume 80 μl). Serial PMCA was then performed in duplicate using the amplification protocol described above.

3. Results

3.1. Effect of L-arginine ethylester on amplification of L-BSE PrP^{Sc}

We examined the amplification efficiency of PMCA, using the mouse PrP^{C} substrate and brain homogenate of L-BSE prion-infected macaque as the PrP^{Sc} seed. In contrast to the amplification of C-BSE PrP^{Sc}, efficient amplification was not achieved even in the presence of polyanions such as Poly-A [25], sulfated dextran [18], heparin [26], and PPS. PPS was found to be an effective agent for amplification of macaque C-BSE PrP^{Sc} in our screening of polyanions (unpublished work). The possible additive effects by the combined use of these polyanions were also examined, but various combinations of polyanions were not quite effective for amplification of L-BSE PrP^{Sc}. For example, in the presence of polyanion cocktail containing heparin, Poly-A and PPS, only weak PrP^{Sc} signal was detected in the brain homogenate diluted to 10^{-3} even after two rounds of amplification (Fig. 1).

Other factors affecting PMCA reaction, such as ultrasonic condition and chelating agent dependency, were also evaluated, but none of the changes examined led to a significant improvement in amplification efficiency. We further investigated the validity of protein denaturants and stabilizing agents, because the amplification deficiency of L-BSE PrP^{Sc} may be due to folded structures that were not suitable for *in vitro* amplification by sonication. Among these reagents, we found that LAE, an arginine derivative known as a powerful inhibitor for protein aggregation [27], is the most effective reagent for amplification of L-BSE PrP^{Sc}. Although LAE alone did not work well enough to induce amplification of L-BSE PrP^{Sc} (data not shown), it strongly promoted the amplification of L-BSE PrP^{Sc} in the range of 12.5–25 mM in the presence of the polyanion cocktail (Fig. 1). In other words, amplification of PrP^{Sc} was achieved in samples diluted up to 10^{-5} after one round of amplification, and the PrP^{Sc} signals were significantly intensified at the next round of amplification. However, LAE had little or no effect on amplification at higher molarity (50–100 mM).

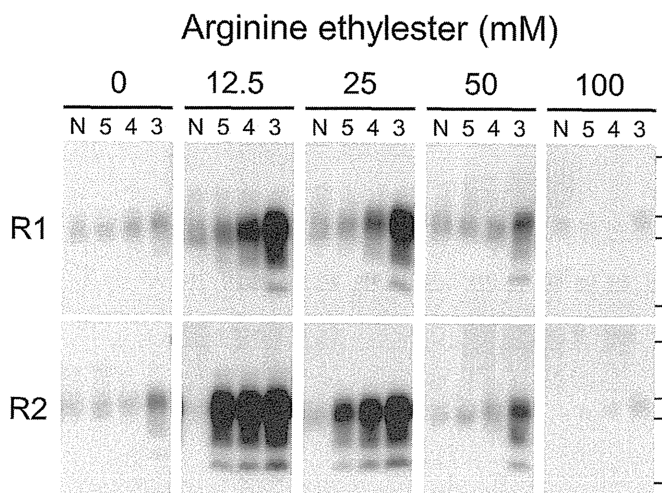

Fig. 1. Amplification of macaque L-BSE PrP^{Sc} using normal mouse brain homogenates. The PrP^{Sc} seed (10% brain homogenate of L-BSE-affected cynomolgus macaque #14) was diluted to 10^{-3} (3) to 10^{-5} (5) in normal mouse brain homogenates containing 100 μg/ml heparin, 100 μg/ml Poly-A and 0.05% PPS. The diluted samples were amplified in the presence (12.5–100 mM) or absence (0) of LAE. The amplified samples were analyzed after each round of amplification (R1–R2) by WB after PK digestion. "N" designates unseeded control samples, which were treated similarly, but containing only the PrP^{C} substrate. Horizontal lines indicate the positions of molecular weight markers corresponding to 37, 25, 20, and 15 kDa.

3.2. Detection sensitivity of L-BSE PrP^{Sc}

On the basis of our preliminary experiments, the optimal concentration of LAE was estimated to be 12.5 mM; therefore, we used 12.5 mM LAE for subsequent experiments. In addition, we confirmed that the most effective amplification was achieved by the combined use of three polyanions, heparin (100 μg/ml), Poly-A (100 μg/ml), and PPS (0.05%), in the presence of 12.5 mM LAE. To determine the detection limit of our novel PMCA technique, 10% brain homogenates of an experimentally infected macaque #14 was serially diluted with the mouse PrP^{C} substrate containing the polyanion cocktail and LAE and amplified. PrP^{Sc} signal was detected in all the quadruplicate samples diluted up to 10^{-9} after five rounds of amplification (Fig. 2). Moreover, PrP^{Sc} signal was detected in one of the quadruplicate samples diluted to 10^{-10} after six or seven rounds of amplification. However, no typical PrP^{Sc} signal was detected in the more extreme dilution, even after eight rounds of amplification. The generation of spontaneous PrP^{Sc} was not observed in the quadruplicate samples that contained only the PrP^{C} substrate following eight rounds of amplification.

3.3. PrP^{Sc} levels in bodily fluids

Neurological clinical signs of the disease, such as tremors and paralysis, appeared in the macaques after latent periods of 486 (#22) and 407 (#23) days. The incubation periods were shorted than those of the primary passaged macaques [22], but the neurological symptoms slowly progressed in these secondary passage macaques.

The presence or absence of PrP^{Sc} in the bodily fluids is summarized in Fig. 3 on the basis of the detection results obtained after eight rounds of amplification (Fig. 4A). There were differences in the levels of PrP^{Sc} in these bodily fluids, and PrP^{Sc} signals were detectable after three–eight rounds of amplification. The complete sets of amplification results are given in the supplementary figures (Fig. S1 for #22, Fig. S2 for #23). After the onset of clinical signs, the presence of PrP^{Sc} in the CSF, saliva, and urine samples was confirmed in both macaques. Furthermore, PrP^{Sc} was also detectable in the CSF samples collected 94–91 days before disease onset. The significant finding is that salivary PrP^{Sc} were detected in the sample collected 150 days before disease onset in macaque #22. Although the time of positive conversion for salivary PrP^{Sc} in this macaque was not clear, PrP^{Sc} was continuously detected in one of the duplicate samples until the dissection. In addition, urinary PrP^{Sc} was detected in the sample obtained 92 days before disease onset in macaque #23. With regard to plasma samples, one of the duplicate samples collected 111days after disease onset became positive for PrP^{Sc} after four rounds of amplification in macaque #23. However, no PrP^{Sc} was detected in any of the plasma samples collected during the experimental period in macaque #22. No typical PrP^{Sc} signal was observed in samples that contained only the PrP^{C} substrate (Ns) or samples that contained concentrated bodily fluids from normal macaques (N1 and N2, Fig. 4B and Fig. S3).

4. Discussion

In this study, we developed an ultra-efficient PMCA technique for amplifying PrP^{Sc} derived from L-BSE prion-infected cynomolgus macaques by using mouse brain homogenates with polyanions and

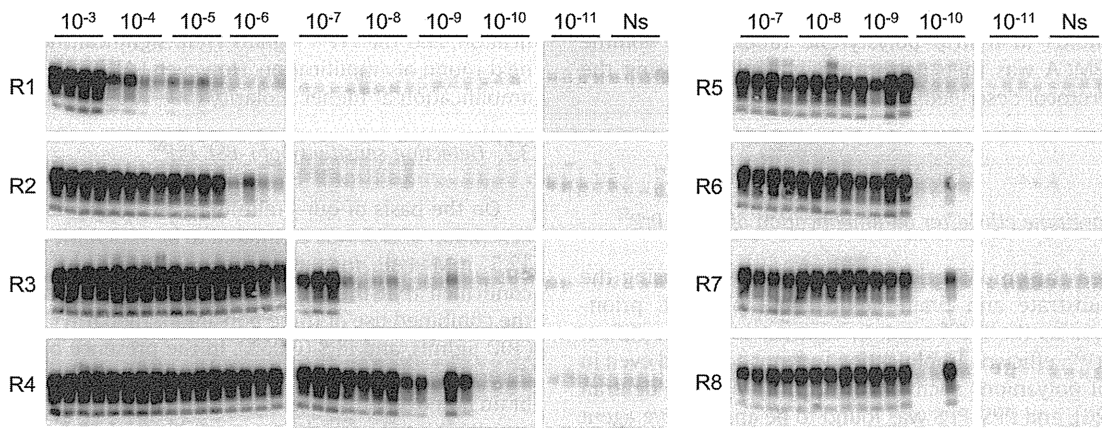

Fig. 2. Detection sensitivity for cynomolgus macaque L-BSE PrP^Sc^. The PrP^Sc^ seed was diluted to 10^{-3}–10^{-11} with the PrP^C^ substrate (10% normal mouse brain homogenate), and the quadruplicate samples were serially amplified in the presence of the polyanion cocktail and 12.5 mM LAE. The amplified samples were analyzed after each round of amplification (R1–R8) by WB after PK digestion. The samples diluted to 10^{-3}–10^{-6} were not amplified after five rounds of amplification because sufficient amount of PrP^Sc^ was produced in all the quadruplicate samples. "Ns" designates unseeded control samples, which were treated similarly, but containing only the PrP^C^ substrate.

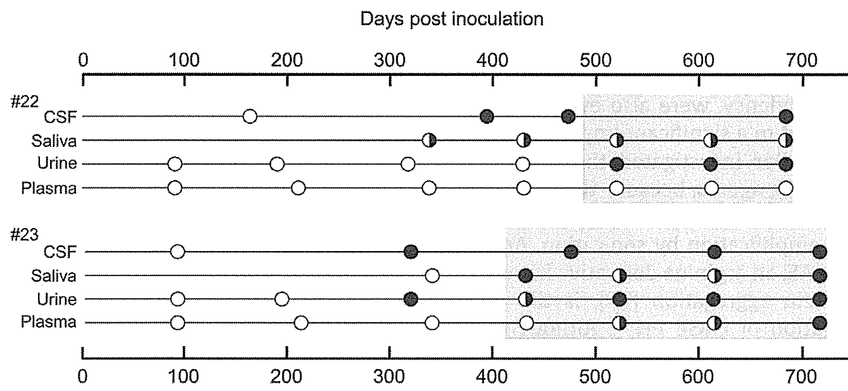

Fig. 3. Schematic illustration for the appearance of PrP^Sc^ in the bodily fluids of two L-BSE prion-infected macaques. After intracerebral inoculation, the presence of PrP^Sc^ in CSF, saliva, urine and plasma samples was examined by serial PMCA during the asymptomatic (circles with white back ground) and clinical stages (circles with gray back ground). Positive ratio of duplicate samples after eight rounds of amplification was shown as open circle (0%), closed semicircle (50%) and closed circle (100%).

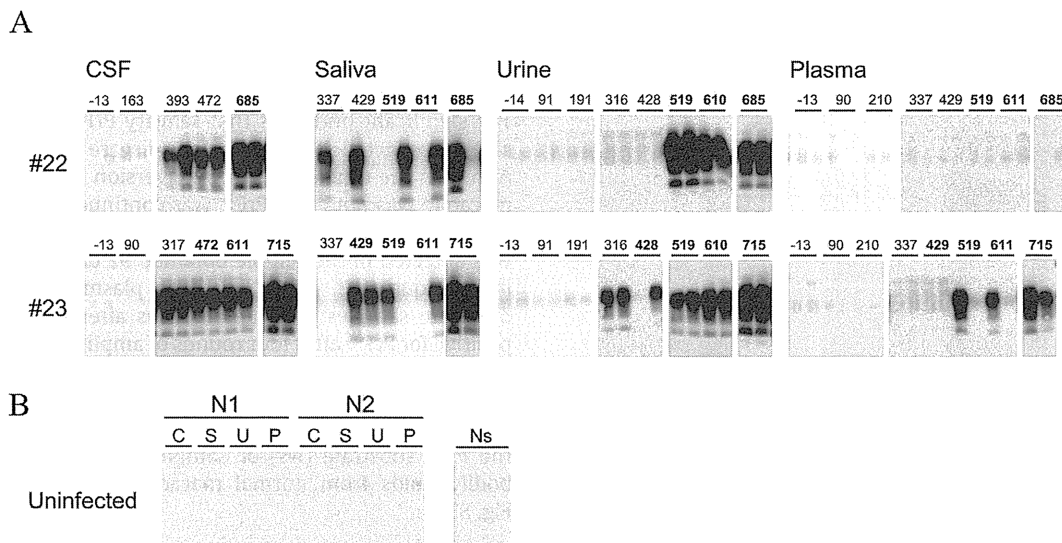

Fig. 4. Detection of PrP^Sc^ in the bodily fluids of L-BSE prion-infected macaques. CSF, saliva, urine, and plasma samples were collected at several points after intracerebral inoculation. (A) Duplicate samples from two L-BSE prion-infected macaques (#22 and #23) were analyzed by WB following digestion with PK. The results of final round (R8) of amplification are shown. The complete set of results of each round of amplification (R1–R8) is given in Supplementary figures. (B) PrP^Sc^ was also evaluated in bodily fluids (C, CSF; S, saliva; U, urine; P, plasma) from uninfected control macaques (N1 and N2). Numerals on the blots represent days post inoculation (dpi), and dpi written in boldface represent the clinical stage of the disease. Negative values represent days before inoculation. Ns: No seed samples.

LAE as a PrP^C substrate. We first proved the presence of PrP^{Sc} in the bodily fluids, including CSF, saliva, urine, and plasma, of the L-BSE-infected macaques by PMCA and then showed that the L-BSE PrP^{Sc} was detectable in saliva and urine samples during the pre-clinical phase of the disease after intracerebral inoculation.

To establish an efficient method to amplify PrP^{Sc} derived from animals with L-BSE, we examined the effects of various additives on the PMCA reaction: protein denaturants or stabilizers such as guanidine, urea, polyamines (spermine and spermidine), high-molecular weight compounds (dextran and polyethylene glycol), trehalose, and L-arginine and its derivatives. Among these additives, LAE was the most effective in enhancing the *in vitro* amplification of macaque L-BSE PrP^{Sc} in the presence of polyanions such as heparin, Poly-A, and PPS. L-arginine was not effective enough whereas an L-arginine methylester compound significantly, but less effectively than LAE, induced amplification of L-BSE PrP^{Sc} in combination with the polyanion cocktail (unpublished work). Therefore, the introduced hydrophobic end on the carboxyl group may play an important role in the amplification of L-BSE PrP^{Sc}.

The functional mechanism of LAE is thought to be different from that of polyanions, which may act as cofactors required to facilitate the propagation of PrP^{Sc} by stabilizing interactions between PrP^{Sc} and PrP^C [18]. L-Arginine enhances the solubility of aggregated molecules, thereby increasing refolding by decreasing aggregation [28,29]. Furthermore, LAE prevented heat-induced aggregation of lysosome more effectively than arginine [27]. The known effect of LAE and the finding that LAE enhanced PrP^{Sc} formation are seemingly contradictory with each other. Hydrophobic side chains of prion protein, which gather inside of a normal PrP^C molecule, become exposed outside of PrP^{Sc} by structural conversion, leading to clustering to form PrP^{Sc} aggregates. LAE may bind preferentially to these misfolded molecules by the hydrophobic ethyl group, and may accelerate the rate of structural conversion by acting as a regulatory factor that prevents excessive aggregation of existing PrP^{Sc} molecules. Further study is necessary to clarify the precise mechanism of LAE in the amplification of L-BSE PrP^{Sc}.

The PMCA technique has been used to identify PrP^{Sc} in a variety of bodily fluids in prion-infected animals and humans [30]. In particular, several reports have described the successful detection of PrP^{Sc} in blood or urine of humans with vCJD [31–33]. In addition to urine, the present study revealed the existence of PrP^{Sc} in the saliva of primates. Salivary or urinary PrP^{Sc} were detected in the samples collected before disease onset in the macaques. Although a limited number of macaques were analyzed in this study, the findings indicate that a non-invasive test for early diagnosis may be developed using this nonhuman primate model. We are now conducting experiments analyzing oral transmission of the L-BSE prion from L-BSE prion-infected cattle. The method developed in this study may be useful in furthering the understanding of leakage of PrP^{Sc} into bodily fluids in nonhuman primate models.

Author contributions

F.O., and H.S conceived and designed the experiments. Y.M., F.O., N.S., and H.S. performed the experiments. Y.M. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.01.105.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.01.105.

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【研究紹介】

道総研畜産試験場における非定型BSEに関する研究

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1．はじめに

牛海綿状脳症(BSE：Bovine Spongiform Encephalopathy)は、羊のスクレイピー、シカの慢性消耗症(CWD)やヒトのクロイツフェルト・ヤコブ病(CJD)などと共に「プリオン病」と呼ばれ、中枢神経組織における空胞変性病変と異常プリオンタンパク質(PrP^{Sc})の蓄積を特徴とする致死性神経疾患である。またBSEは、ヒトの変異型CJDの原因となるなど人獣共通感染症として公衆衛生上、重要な疾患である。

BSEは、1986年に英国で初めて確認され、1992年には37,280頭の発生が見られた。また英国から欧州諸国、北アメリカおよび日本に拡散した。各国がBSE対策を実施した結果、近年BSEの発生頭数は年々減少しており、2013年では７頭、2014年では12頭となった（国際獣疫事務局：OIE公表）(図１)。わが国のBSEでは、2001年９月に第１例目のBSEが報告され、大きな社会問題となった。しかしその後の飼料製造工程での混入防止策を含めた反芻家畜への動物性飼料の給与禁止、食肉処理場における特定危険部位の除去やBSE検査の実施等の行政措置や生産者・畜産関係者の努力が功を奏し、2009年３月の36例目の患畜を最後にBSE患畜は確認されず、終息状態となっている。また過去11年間に生まれた牛からBSE患畜が出ていないことなどから、2013年にOIEのBSEリスクステータス「BSEのリスクを無視できる国」に承認されたところである。BSE発生以来、継続していた食肉衛生検査でのBSE全頭検査も、リスク評価機関による評価を踏まえ、段階的に対象月齢を変更し、また死亡牛検査もこの度対象月齢が変更され、現在はともに48カ月齢超の牛が対象となっている。

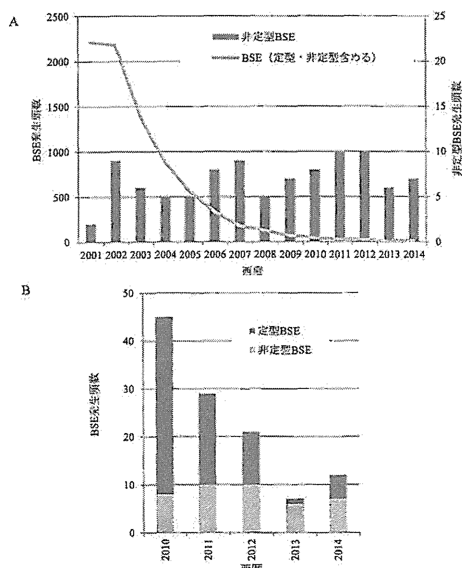

図１　世界のBSE発生頭数の推移

A．BSE発生頭数（定型・非定型を含む）と非定型BSE発生頭数（2001～2014）
B．定型BSEと非定型BSEの発生頭数の推移(2010～2014)

当初、BSEは単一のプリオン株に起因すると考えられてきたが、定型BSE発生拡大に伴い各国でBSE検査が行われた結果、2000年代に入り、これまでの「定型BSE」と性状の異なる「非定型BSE」が欧州、北米、南米、日本で散発的に報告されている。非定型BSEは、ウエスタンブロット（WB）法によるBSE検査でPrP^{Sc}の泳動パターンが定型BSEと異なり、L型およびH型の２つの型が報告されている。H型は、タンパク質分解酵素プロテイナーゼK(PK)で消化処理した後のPrP^{Sc}の分子量が、定型BSEよりも大きく、WBのバンドの位置が高く検出され、L型は分子量が小さく、WBのバンドの位置が低く検出される。米国で確認された非定型BSE（H型）１例にプリオンタンパク質遺伝子の変異が見つかっている他は、患畜のPrPにアミノ酸配列の

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違いは見られていない。この分子量の違いは、PK処理に対する抵抗性部位の差によるものであり、アミノ酸配列に差がないことから立体構造の違いにより生じるものと推察されている。わが国においても、BSE患畜36例のうち2例（第8例目と第24例目）が非定型BSEであった。非定型BSEのほとんどは死後検査で確認されているため、臨床症状や農場段階での診断に関する情報はほとんどない。非定型BSEの発生機序が不明であり、ヒト型PrP遺伝子組換えマウスや霊長類への脳内接種試験によりヒトへの感染リスクが示唆されていることから、BSE問題の残された課題となっている。

道総研畜産試験場における定型BSEに関する研究について、先に本誌3月号（第59巻第3号）にて紹介した。道総研畜産試験場では、さらに非定型BSEの感染実験牛を用いた非定型BSEの診断技術の開発と発生要因の解明について研究を行っており、本稿ではその研究成果について紹介するとともに、非定型BSEについて若干解説する。

2．畜産試験場における非定型BSE研究

試験1．非定型BSE感染牛の臨床症状

目的

非定型BSE（L型）と診断された国内24例目のBSE患畜（BSE/JP24）[1]の脳を接種した非定型BSE感染牛を作出し、非定型BSEにおけるプリオンの接種から臨床症状の発現までの期間、接種から起立不能等のため飼育困難になるまでの期間および臨床症状について定型BSE感染牛と比較する。

方法

BSE非発生農場で出生したホルスタイン種雌牛14頭および黒毛和種雌牛6頭を用いた。BSE/JP24の10%脳乳剤をホルスタイン種7頭および黒毛和種3頭に、定型BSE感染牛の10%脳乳剤をホルスタイン種7頭および黒毛和種3頭に、それぞれ2～4カ月齢時に1 ml ずつ脳内に接種した。BSE感染牛の飼育は専用隔離牛舎（動物バイオセーフティレベル1）で行った。BSE感染牛の観察は、BSEの臨床症状検査を実施し、すなわち頭部を低くする等の姿勢や異常行動、歩様や走行姿勢の変化、音に対する過剰反応、動くものに対する過剰反応を毎週1回観察した。

非定型BSE感染牛の2頭（接種後9カ月）および定型BSE感染牛の5頭（接種後12～22カ月）は、臨床症状を発現する前に病理解剖を行った。その他の13頭は、歩様の変化や音への過剰反応、起立困難などのBSEの臨床症状が現れた後、病理解剖した。脳および末梢組織におけるPrP^{Sc}の分布は、WB法を用いて解析した。

表1．非定型および定型BSE感染牛の臨床症状の経過

	牛No.	品種	初発の臨床症状	接種後の月数 9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	解剖前の状態
非定型（L型）BSE感染牛	A1[1]	Hol	なし	□−																症状なし
	A2[1]	Hol	なし	□−																症状なし
	A3[2]	Hol	（起立困難）	−	−	■−														起立不能
	A4	Hol	歩様の変化	−	−	−	■+													運動失調
	A5	Hol	歩様の変化	−	−	−	+	■+												運動失調
	A6	Hol	音に過剰反応	−	−	+	+	■+												運動失調
	A7[2]	Hol	（起立困難）	−	−	−	−	−	−	−	■−									起立不能
	A8	JB	歩様の変化	−	−	−	−	−	−	■+										運動失調
	A9	JB	音に過剰反応	−	−	+	+	+	+	+	■+									運動失調
	A10	JB	音に過剰反応	−	−	−	−	−	+	+	■+									運動失調
定型BSE感染牛	C1[1]	Hol	なし	−	−	−	□−													症状なし
	C2[1]	Hol	なし	−	−	−	□−													症状なし
	C3[1]	Hol	なし	−	−	−	−	−	−	−	□−									症状なし
	C4[1]	Hol	なし	−	−	−	−	−	−	−	−	−	□−							症状なし
	C5[3]	Hol	起立姿勢・歩様の変化	−	−	−	−	−	−	−	−	−	+	+	■+					姿勢異常
	C6	Hol	起立姿勢・歩様の変化	−	−	−	−	−	−	−	−	−	+	+	+	+	+	■+		起立不能
	C7	Hol	歩様の変化	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	■+	起立不能
	C8	JB	音に過剰反応	−	−	−	−	−	−	−	−	−	−	■+						運動失調
	C9	JB	音に過剰反応	−	−	−	−	−	−	−	−	−	−	−	−	+	■+			運動失調
	C10[1]	JB	なし	−	−	−	−	−	−	−	−	−	−	−	−	−	□−			症状なし

＊起立不能等により飼養困難となった非定型BSE感染牛：8頭（A3～A10）、定型BSE感染牛：5頭（C5～C9）について比較した。
1）臨床症状が現れる前に試験殺し解剖した。
2）臨床症状検査に対する反応がなく、突然起立困難となった。
3）臨床症状確認後、接種後20カ月にて計画的に病理解剖した。
＊品種　Hol：ホルスタイン種、JB：黒毛和種
＊−：臨床症状なし、＋：臨床症状あり、□：病理解剖（所見なし）、■：病理解剖（所見あり）

結果

脳内接種による非定型および定型BSE感染牛の臨床上の経過と病理解剖までの期間を表1に示した。

非定型BSE感染牛の初期の臨床症状として、脳内接種から11～16カ月経過した後、歩様の異常（後肢のふらつき）が3頭、音への過剰反応が3頭に見られた。姿勢・行動の変化および動く物に対する過剰反応は見られず、また複数の臨床症状を呈した牛はいなかった。また2頭は、これらの臨床症状の検査項目のいずれでも異常が観察されなかったが、突然起立困難となった。定型BSE感染牛では、脳内接種から18～21カ月後に、佇立姿勢や歩様の異常、音への過剰反応などの症状が見られ、複数の症状を示す牛もあった。非定型BSE感染牛は、定型BSE感染牛と比較して臨床症状が不明瞭であった。脳内接種による非定型BSE感染牛が飼育困難になるまでの期間は、脳内接種からおよそ11～16カ月後であり、定型BSE感染牛の19～24カ月後よりも約8カ月早かった。

考察

非定型BSE感染牛の脳内接種から臨床症状の発現までの期間および飼育困難になるまでの期間は、定型BSE感染牛と比較して約8カ月短く、非定型BSE（L型）プリオンは、定型BSEプリオンと比較して、牛に対する病原性が強いと考えられた。非定型BSE感染牛は、臨床症状の検査項目のいずれでも異常の見られない個体がいるなど、定型BSE感染牛と比較して臨床症状は不明瞭であった。非定型BSEのための臨床症状の検査方法の検討が必要と考えられた。

非定型および定型BSE感染牛のいずれにおいても、黒毛和種とホルスタイン種の間に臨床症状の差は見られなかった。

試験2．非定型BSE感染牛のPrP^{Sc}の体内分布

目的

非定型BSE感染牛の脳および末梢組織におけるPrP^{Sc}の蓄積時期と分布を明らかにする。

方法

試験1の20頭を用いた。非定型BSE感染牛のうち2頭（接種後9カ月）および定型BSE感染牛のうち5頭は、臨床症状を発現する前に病理解剖行った。病理解剖は、供試牛を鎮静および麻酔下で安楽殺し、各組織を採取した。採取した脳および末梢組織は、Hayashiら[2]またはShimadaら[3]の方法により処理し、HRP標識T2マウスモノクローナル抗体を用いたWB法によりPrP^{Sc}を検出した。WB法の結果の判定は、脳では、陽性対照であるマウススクレイピー脳1.6mg組織等量と比較し、発光強度が強い検体を＋＋、PrP^{Sc}を確認できるが発光強度がそれ以下の検体を＋、PrP^{Sc}が認められなかった検体を－と判定した。また末梢組織においては、PrP^{Sc}を認めた検体を＋、認められなかった検体を－と判定した。

結果

脳各部位のPrP^{Sc}の解析を行った結果、BSE感染脳乳剤を接種したすべての牛の脳幹部からPrP^{Sc}が検出された（表2）。非定型BSEを接種し9カ月後に解剖した牛では、脳幹部に加え、嗅脚、線条体および視床などの部位においてもPrP^{Sc}が検出された。非定型BSE接種後12カ月以降の牛では、脳のほぼ全域からPrP^{Sc}が検

表2．脳内接種による非定型および定型BSE感染牛の脳へのPrP^{Sc}の蓄積

		非定型BSE（L型）										定型BSE									
	牛No.	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
	品種	Hol	Hol	Hol	Hol	Hol	Hol	Hol	JB	JB	JB	Hol	Hol	Hol	Hol	Hol	Hol	Hol	JB	JB	JB
部位	接種後月数	9	9	11	12	13	13	16	15	16	16	10	12	16	18	20	23	24	19	22	22
嗅脚		++	++	++	++	++	++	++	++	++	++	−	−	−	−	++	++	++	++	++	++
前頭葉皮質		−	−	−	++	+	++	++	++	++	++	−	−	−	−	++	++	++	++	++	++
前頭葉髄質		−	−	−	++	+	−	++	−	++	−	−	−	−	−	++	+	+	+	+	+
線条体		+	+	−	++	++	++	++	++	++	++	−	−	++	+	++	++	++	++	++	++
視床		++	+	+	++	++	++	++	++	++	++	−	−	−	+	++	++	++	++	++	++
頭頂葉皮質		−	−	−	+	+	++	++	++	++	++	−	−	−	−	++	++	++	++	+	++
頭頂葉髄質		−	−	−	+	−	−	++	−	−	−	−	−	−	−	++	++	++	++	+	+
海馬		−	+	−	+	−	++	++	++	++	++	−	−	−	+	++	++	++	++	++	++
小脳皮質		−	+	−	++	−	++	++	++	++	++	−	−	−	++	+	++	++	++	+	+
小脳髄質		++	++	−	++	++	++	++	++	++	++	−	+	−	++	+	++	++	++	++	++
中脳		++	++	++	++	++	++	++	++	++	++	+	+	++	++	++	++	++	++	++	++
橋		++	++	++	++	++	++	++	++	++	++	+	+	++	++	++	++	++	++	++	++
延髄閂		++	++	++	++	++	++	++	++	++	++	+	+	++	++	++	++	++	++	++	++

出された。定型BSEを接種し10カ月後の牛では、PrP^{Sc}は脳幹部のみに検出され、接種後19カ月以降より、脳のほぼ全域からPrP^{Sc}が検出された。なお、非定型BSE感染牛では、WB法により検出されたPrP^{Sc}が、接種したBSE/JP24と同じ糖鎖パターン、すなわち1糖鎖型優位を示した。

末梢組織では、接種後9、11および12カ月では頭部の末梢神経に、接種後13カ月で体幹にある星状神経節、接種後16カ月では腕神経叢など前肢の末梢神経にPrP^{Sc}が検出され、定型BSE感染牛と同様に、経過に伴い遠心性にPrP^{Sc}が伝播することが示唆された（**表3**）。また非定型BSE感染牛では、リンパ系組織等からはPrP^{Sc}は検出されず、定型BSE感染牛と同様の結果であった。

考察

非定型BSE感染牛では、定型BSE感染牛と比較して、脳においてPrP^{Sc}が早期に蓄積し、早く伝播すると考えられた。試験1の結果と同様に、非定型BSEのPrP^{Sc}は、定型PrP^{Sc}と比較して病原性が強いと考えられた。非定型および定型BSE感染牛のいずれにおいても飼育困難になり病理解剖を行った時点では、PrP^{Sc}は脳のほぼ全域に分布し、分布する部位には差は見られなかった。また、脳における分布の部位には、黒毛和種とホルスタイン種に差は見られなかった。非定型BSE感染牛では、経過に伴い、遠心性に末梢神経組織にPrP^{Sc}が伝播することが示唆された。また、リンパ系組織等からはPrP^{Sc}は検出されなかったことから、PrP^{Sc}が神経系組織以外へ伝播する可能性は低いと考えられた。これらのことは定型BSE感染牛と同様であった。Iwamaruら[4]は脳内接種の非定型BSE感染牛において、リンパ系組織、筋肉などからはPrP^{Sc}は検出されないことを報告しており、PrP^{Sc}が神経系組織以外へ伝播する可能性は低いと考えられた。非定型および定型BSEの末梢組織へのPrP^{Sc}の蓄積は、脳におけるPrP^{Sc}の蓄積時期の差を反映していると考えられた。

3．まとめ

BSEの発生状況について、OIEでは定型と非定型を分けた報告を加盟国に求めていないため、非定型BSEの正確な発生数はまとめられていない。そこで、OIE、欧州食品安全機関（European Food Safety Authority：EFSA）および各国の報告等から非定型BSEの発生数を集計したところ、2015年4月末日現在までに98例が報告されている（**表4**）。前述のように世界各国がBSE対策を実施した結果、定型BSEの発生頭数は年々減少しているものの、非定型BSEは、毎年5～10頭の範囲で報告されている。発生頭数が少ないことと各国のこれまでのBSE検査の実施過程に違いがあることから、比較は容易にできないが、各国の非定型BSEの発生頭数

表3．非定型および定型BSE感染牛の末梢組織におけるプリオンの検出

		非定型BSE							定型BSE				
牛No.		A1	A2	A3	A4	A5	A6	A7	C3	C4	C5	C6	C7
接種後の月数		9	9	11	12	13	13	16	16	18	20	23	24
脊柱	脊髄神経節頸膨大部		−	−	+	+	+	+	+	−	−	+	+
	脊髄神経節腰膨大部	−	−	−		+	+	+	−	−	−	+	+
頭部	下垂体	−	−	+	−	−	−	+	+	−	−	+	+
	視神経	+	+	+	+	+	+	+	−	−	−	+	+
	網膜			+	+	+	+		−	+	+	+	+
	三叉神経節	−	+	+	−	+	+	+	+	+	+	+	+
体幹	交感神経幹	−	−	−	−	−	−	+	−	−	−	−	+
	星状神経節	+	−	−	−	−	+	+	−	−	−	+	+
	横隔神経	−	−		−	−		+	−	−	−	−	+
	腹腔神経節	−	−	−		−		−		−	−	−	−
四肢	腕神経叢	−	−	−	−	−	−	+	−	−	+	+	+
	肩甲上神経	−	−	−	−	−		+	−	−	−	−	+
	正中神経	−	−	−	−	−	−	−		−	+	+	+
	坐骨神経	−	−	−	−	−	+	−	−	−	−	+	−
リンパ	脾臓	−	−	−	−	−	−	−	−	−	−	−	−
	扁桃	−	−	−	−	−	−	−	−	−	−	−	−
	腸間膜リンパ節（回盲部）	−	−		−	−			−				
その他	耳下腺	−	−			−		−					
	回腸	−	−		−	−	−			−	−	−	−
	最長腰筋	−	−			−				−	−	−	
	腰長筋	−	−			−			−		−	−	−
	半腱様筋	−	−			−			−	−	−	−	−

＋：プリオン陽性、－：陰性、空白：未実施

表4．世界各国における定型および非定型 BSE 発生頭数

地域	国名	非定型BSE				定型BSE	牛の飼養頭数（千頭）※1
		H型	L型	未分類	計		
欧州	オーストリア	1	2		3	5	1,977
	デンマーク	0	1		1	15	1,607
	フランス	16	16		32	994	19,006
	ドイツ	2	2		4	417	12,477
	アイルランド	5	0		5	1,650	6,754
	イタリア	0	5		5	139	6,252
	オランダ	1	3		4	84	3,879
	ポーランド	2	12		14	60	5,777
	ポルトガル	1	0		1	1,082	1,498
	スペイン	3	2		5	782	5,813
	スウェーデン	1	0		1	0	1,500
	イギリス	5	4		9	184,616	9,900
	ノルウェー	1	0		1	0	862
	ルーマニア	0	1		1	1	1,989
	スイス※2	1	0	2	3	464	1,565
北米	アメリカ	2	1		3	0	90,769
	カナダ	1	1		2	19	12,305
南米	ブラジル	1	0	1	2	0	211,279
アジア	日本	0	2		2	34	4,172
その他の国※3		0	0		0	204	
計		43	52	3	98	190,566	

※頭数は OIE、EFSA および各国の報告等に基づき算出した。（2015年4月末日現在）
※1：2012年（帝国書院）
※2：スイスはこの他に動物園での zebu 牛の非定型 BSE が報告されている。
※3：BSE 発生国のうち非定型 BSE の発生のない国：ベルギー、チェコ共和国、フィンランド、ギリシア、イスラエル、リヒテンシュタイン、ルクセンブルグ、スロバキア、スロベニア

と定型 BSE の発生頭数または牛の飼養頭数に強い関連性は見いだせない。また L 型と H 型の発生数の比較では、L 型 BSE の発生が多い国（イタリアやポーランド）や H 型の発生が多い国（アイルランド）があり、国ごとに異なる。また米国（カナダからの輸入牛を除く）、ブラジル、スウェーデン、ノルウェーのように、これまで定型 BSE の発生が見られず、非定型 BSE が報告される国もある。スイスでは、L 型と H 型のいずれとも異なる2例が報告されている。アジアにおいては、我が国の8例目（23カ月齢、BSE/JP08）[5]と24例目（168カ月齢、BSE/JP24）の2例の BSE 患畜がそれぞれ非定型 BSE（L 型）と報告されている。

BSE/JP08は、生後23カ月齢でと殺された去勢牛で、採取された脳を牛 PrP 遺伝子組換えマウスに脳内接種しても感染が成立しない程 PrP^{Sc} の蓄積が極微量であった[6]。BSE/JP24は、カナダ、ドイツおよびフランスで発生した L 型 BSE と比較し、PK で消化した PrP^{Sc} の分子量や糖鎖パターンがほぼ同様である。また、それぞれの L 型 BSE プリオンを牛 PrP 遺伝子組換えマウスに脳内接種により継代したところ、3回目の継代では約145日に集束するなど、BSE/JP24は、欧州の L 型 BSE と同様の性質を持つことが明らかとなっている[7]。H 型 BSE の国内発生はこれまでにないが、カナダで発生した H 型 BSE プリオンの脳内接種による牛への感染試験によれば、接種から終末までの期間が560±47日と定型 BSE よりも短く、L 型 BSE よりも長い[8,9]。

定型 BSE は3～8歳の牛に多く発見されたが、非定型 BSE は国内8例目など一部を除いてほとんど8歳以上の高齢牛で見つかっている。しかし脳内接種による非定型 BSE 感染牛では、L 型および H 型共に、PrP^{Sc} の蓄積が早く、臨床症状の発現や飼育困難になるまでの時期も定型 BSE 感染牛と比較して早かった。また非定型 BSE プリオンの牛への経口投与試験については、これまでに経口投与牛の中枢神経組織への PrP^{Sc} の蓄積を確認した報告はない。非定型 BSE の感染・発症機序は未だ明らかではないが、1）定型 BSE と同様に肉骨粉等のプリオンが混入した飼料を摂取することにより感染する、2）飼料摂取とは関係なく孤発性に発生する、などが考えられる。1）の場合、飼料の摂取から中枢神経系への移行に定型 BSE 以上の時間を要するものの脳での増幅・蓄積は早いことが考えられる。また2）の場合では、加齢による細胞レベルの蛋白質代謝異常に起因する

ことなどが考えられる。

以上のように、非定型BSEには不明な点が未だ多くあり、孤発性であることが示唆されている。定型BSEの発生数やOIEのBSEリスクステータスなどを問わず、極めて稀であるものの発生することが懸念される。非定型BSEの発生リスクのある高齢牛は必ずBSE検査を行われることや特定危険部位は全て除去されることから、ヒトへの感染リスクは排除され、畜産物の安全は確保されている。しかしながら、今後、科学的知見に基づいた非定型BSEのリスク評価と効率的なリスク管理を行うため、非定型BSEの発生要因やPrP^{Sc}の蓄積機序を明らかにすることは重要であり、ヒトの孤発性CJDや羊のスクレイピー、シカのCWDを含め、プリオン病の感染・発症機序解明にさらに知見の集積が必要である。

現在、道総研畜産試験場では、脳内接種による非定型BSE感染牛を用いた試験を継続しており、これまでに、歩様と行動量の変化から、非定型BSE感染牛の臨床症状を把握できる可能性を示した。また、非定型BSEの感染初期におけるPrP^{Sc}の経時的な蓄積量の変化を調査する試験を実施中である。さらに、ヒトや牛で加齢に伴い脳内の酵素活性が低下することで不溶性タンパク質が増加すると報告されることから、高齢牛の脳内不溶性タンパク質と非定型BSEの関連性を調査する試験を実施している。これらの研究成果により、今後のBSEのリスク評価と効果的なBSEリスク管理措置の策定に必要なデータを提供することを目指している。

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