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Quantitative analysis of wet-heat inactivation in bovine spongiform encephalopathy

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ABSTRACT

The bovine spongiform encephalopathy (BSE) agent is resistant to conventional microbial inactivation procedures and thus threatens the safety of cattle products and by-products. To obtain information necessary to assess BSE inactivation, we performed quantitative analysis of wet-heat inactivation of infectivity in BSE-infected cattle spinal cords. Using a highly sensitive bioassay, we found that infectivity in BSE cattle macerates fell with increase in temperatures from 133 °C to 150 °C and was not detected in the samples subjected to temperatures above 155 °C. In dry cattle tissues, infectivity was detected even at 170 °C. Thus, BSE infectivity reduces with increase in wet-heat temperatures but is less affected when tissues are dehydrated prior to the wet-heat treatment. The results of the quantitative protein misfolding cyclic amplification assay also demonstrated that the level of the protease-resistant prion protein fell below the bioassay detection limit by wet-heat at 155 °C and higher and could help assess BSE inactivation. Our results show that BSE infectivity is strongly resistant to wet-heat inactivation and that it is necessary to pay attention to BSE decontamination in recycled cattle by-products.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal and progressive neurodegenerative diseases that include bovine spongiform encephalopathy (BSE), Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep and goats, and chronic wasting disease in deer and elk. In TSE-affected animals, abnormal prion proteins (PrP^{Sc}), a disease-related isoform of a host-encoded normal prion protein (PrP^{C}), accumulates in the brain and is partially resistant to proteinase K digestion. According to the prion hypothesis, PrP^{Sc} may be the major component of the TSE infectious agent (also known as prions) [1,2] and mediate the structural conversion of PrP^{C} into PrP^{Sc}. Experimentally, TSE seeding activity that involves this conversion is monitored by the protein misfolding cyclic amplification (PMCA) assay [3].

TSE agents are highly resistant to conventional physical and chemical procedures for inactivating or eliminating microbes. Inactivation efficiency of the agents changes depending on the tissue states (homogenate or macerate), type of heating (wet or dry) or autoclave mechanism (gravity displacement or porous-load) [4]. In addition, the degree of inactivation varies between TSE agent strains [5]. Several papers have reported that the BSE agent causing variant CJD is more resistant to wet-heat inactivation than other agents of CJD and scrapie [6–9] and survives even after treatment at 134 °C for up to 2 h [8, 9]. Incomplete inactivation of BSE infectivity in cattle tissues during rendering was probably one of the major contributory causes of the BSE epizootic. Accordingly the inactivation profile of BSE infectivity in cattle tissues provides important information for assessment of measures for the control and prevention of the disease.

This report describes a quantitative analysis of BSE infectivity in cattle tissues after wet-heat treatment in a gravity displacement autoclave. We investigated the effect of tissue dehydration on wet-heat inactivation by testing wet-heat treatment on BSE cattle tissues with differing water content. After wet-heat treatment of BSE cattle tissues, the infectivity was quantitatively evaluated using a bioassay involving bovinized transgenic mice. We then confirmed the degree of BSE inactivation using the quantitative PMCA assay [10, 11].

2. Materials and methods

2.1. BSE-infected cattle tissue

The spinal cords from 4 cows (codes 3217, 4612, 5087, and 5523) experimentally inoculated with classical BSE [12] were pooled and homogenized without any additional fluid using a

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blender. The material was divided into small aliquots (200 ± 5 mg) and put on glass dishes (10 × 10 mm; diameter × height). Samples of fully hydrated macerate were used without further processing. Semi-dried macerate was obtained by exposing samples to a dry-air oven at 65 °C for 2 h, resulting in a 30% weight loss. Fully dried macerates were obtained by exposing samples to 65 °C for 24 h in the dry air oven, resulting in 70% weight loss. The BSE infectivity of one macerate sample was measured by the end point titration assay in bovinized transgenic mice (Table 1).

2.2. Wet-heat treatment of samples

Samples placed in glassware were directly transferred to a container (5 mL volume) and autoclaved for 20 min in a TEM-D300M system (Taiatsu Techno Corporation, Tokyo, Japan) as previously described [13]. Temperature and pressure were monitored during wet-heat treatment. The exposure time was counted from the point the target temperature ranged from 133 °C to 170 °C (see Table 2). Treatments were performed in duplicate or triplicate. After wet-heat treatment, the sample was transferred to a new tube and homogenized in 10% (w/v) phosphate-buffered saline (PBS), based on the initial macerate weight. All homogenates were stored at −80 °C and sonicated prior to analysis.

2.3. Bioassay in bovinized transgenic mice

The bovinized transgenic mouse Ki+Tg#40, carrying the open reading frame of bovine PrP with 6 octarepeats, is homozygous for both transgenes of knock-in and random insertion, and expresses 12-fold more bovine PrP^C in the brain than a bovinized knock-in mouse but does not express mouse PrP^C (data not shown) [14]. The mice were intracerebrally inoculated with 20 μL of 10% homogenates of the treated and untreated samples. To calculate the 50% lethal dose in 1 g equivalent cattle tissue (LD_{50}/g), the same volumes of 10-fold dilutions of untreated BSE macerate were inoculated into the right hemisphere. The bioassay also yielded a standard curve for the relationship between incubation period and LD_{50}/g. The mice were housed in a biosafety level 3 room of our animal facility and were monitored every 2 d; they were euthanized once they fell ill or were observed until 850 d post inoculation. The incubation period (in days) from inoculation to euthanasia has been provided as the mean and standard deviation value for BSE-affected mice in each group.

To confirm BSE transmission, the brains of the euthanized mice were subjected to histopathology analysis [15] and Western blotting with the anti-PrP monoclonal antibody 6H4 (Prionics AG, Switzerland). The mouse brain was sliced in half sagittally; the right brain was fixed in formalin for histopathology analysis and the other half was frozen at −80 °C for Western blot analysis. BSE-affected mice were identified by immunohistochemical analysis of PrP^{Sc} deposits in the mouse brain or proteinase K resistant PrP^{Sc} on Western blots.

Table 1
End-point titration assay of untreated BSE macerate.

Dilution factor	n/n_0[a]	Incubation period (mean days ± SD)
−1[b]	7/7	321 ± 26
−2	6/6	326 ± 30
−3	5/5	384 ± 17
−4	5/6	424 ± 66
−5	0/6	–[c]
−6	0/6	–

[a] Number of BSE-affected mice per population of inoculated mice.
[b] 10% homogenate of the BSE macerate.
[c] Mice who did not have BSE until 850 d post inoculation.

All animal experiments were approved by the Committee of Animal Experiment, National Institute of Animal Health, and were performed according to the Guideline for Animal Experiment of the Ministry of Agriculture, Forestry, and Fisheries of Japan.

2.4. Quantitative PMCA

The treated and untreated 10% cattle macerate homogenate inocula were considered −1 log dilution and were then serially diluted (10-fold). The diluted samples (2 μL) with 78 μL of PrP^C substrate containing 0.5% potassium dextran sulfate were subjected to PMCA with 40 cycles of sonication (one pulse oscillation for 5 s, repeated five times at 1 s intervals), followed by incubation at 37 °C for 1 h with agitation [11]. The PMCA product (16 μL) with 64 μL of the substrate was used to initiate each subsequent round, and the process was repeated for a total of 4 rounds. The PMCA products were assayed by Western blotting for the detection of proteinase K-resistant PrP^{Sc}. The assays were performed in quadruplicate and were calculated as the log dose of 50% available PMCA seeding activity per 1 g wet weight equivalent (indicated as log $PMCA_{50}$/g) by the Spearman–Kärber method, using the positive and negative seeding activity ratios in each 10-fold dilution.

3. Results

3.1. Infectivity titer of the untreated BSE macerate

Ki+Tg#40 mice developed mouse BSE at 321 d post inoculation with 10% untreated BSE homogenate. The incubation period was prolonged and the rate of mice affected with BSE decreased with increasing dilution ratios (Table 1). The infectivity detection limit was −4 log dilution of the untreated BSE macerate. The infectivity titer of the untreated BSE macerate was calculated as 6.03-log LD_{50}/g by the Spearman–Kärber method by using the ratios of positive and negative mice in each dilution group.

3.2. Bioassay of BSE cattle tissues using bovinized transgenic mice

Ki+Tg#40 mice were affected with BSE when inoculated with macerate treated at 133–150 °C. The incubation periods increased with increase in processing temperature (Table 2). No mice were infected after inoculation with macerate heated at or above 155 °C. Mice inoculated with BSE semidry materials that were treated at 133–155 °C were affected with BSE, but inocula treated at ⩾160 °C did not produce disease. The incubation periods increased with increase in processing temperature. In the case of dry BSE materials, mice were affected with BSE even at 170 °C in 1 of the 3 samples tested. The incubation periods were occasionally shorter than those for untreated material, with processing temperatures from 133 °C to 155 °C. No significant histological differences between infected brains were observed (data not shown).

As shown in **Fig 1**, the available data indicate that BSE infectivity was stable until near a temperature of 130 °C, but then in rapidly reduced with increasing temperature. Dried material was not substantially inactivated until a temperature in excess of 150 °C was obtained, but higher temperatures rapidly reduced infectivity to undetectable levels above 170 °C. Infectivity titers were calculated from incubation periods using the dose response curve obtained from the titration of the macerate (6.03-log LD_{50}/g) (Table 1). The calculated titers for macerate heated at 133 °C ranged from 4.03-log LD_{50}/g and 4.70-log LD_{50}/g, and fell to undetecatable levels at 155 °C. Infectivity in treated semidry material also decreased with increasing temperature, compared to 5.03-log LD_{50}/g in the untreated semidry tissue, and reached the detection limit at 155 °C. In the case of BSE dry materials, infectivity after

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