

Transgenic mouse brains for the evaluation and quality control of BSE tests

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Abstract

Rapid BSE tests are widely used diagnostics in veterinary medicine and more than 11 million tests are applied worldwide. The evaluation of new rapid BSE tests and the quality assurance of approved BSE tests pose a challenge owing to the natural scarcity of BSE-infected bovine brainstems and regional variations in prion titer. Transgenic mice expressing bovine prion protein (Tg4092) offer an alternative approach to these problems. To determine whether BSE-infected Tg4092 mouse brains could serve as a general standard for rapid BSE tests, we inoculated Tg4092 mice intracerebrally with BSE prions, harvested brains at defined time points post-infection and analyzed cerebral hemispheres with several approved rapid BSE tests. The results show that *de novo* formation of the disease-causing prion protein isoform, PrP^{Sc}, can be monitored during the course of infection. We demonstrate that BSE-infected Tg4092 mouse brains provide a renewable and controllable source of reference samples and suggest that such samples can generally

be used for the evaluation and quality control of rapid BSE tests.

Keywords: bovine spongiform encephalopathy; prion protein; rapid BSE test; transgenic mouse.

Introduction

Rapid tests for the *post mortem* diagnosis of bovine spongiform encephalopathy (BSE) or ‘mad cow disease’ are used more than 11 million times annually worldwide. In the European Union, these rapid BSE tests were evaluated for use by the European Commission’s (EC) Institute for Reference Materials and Measurements, resulting in 12 officially approved tests (EC, 2001; Philipp and Vodrazka, 2004). However, the continuing evaluation of new rapid tests for BSE prions and the quality control of approved BSE tests are confronted with two problems: the poor availability of BSE-infected bovine brainstems and the heterogeneity in prion titer along bovine brainstems (Safar et al., 2002).

Transgenic mice expressing bovine prion protein, designated Tg(BoPrP^{+/+})4092/*Prnp*^{0/0} mice (Scott et al., 1999), or Tg4092 mice for simplicity, are an alternative for overcoming these problems. We therefore inoculated Tg4092 mice intracerebrally with BSE prions, harvested their brains during the infection, and subjected large numbers of cerebral hemispheres to analysis with five different rapid BSE tests.

We found that such samples are generally suitable for assessing rapid BSE tests, and that *de novo* formation of the disease-causing prion protein isoform, designated PrP^{Sc}, can be monitored during the course of infection. All five rapid BSE tests initially detected nascent PrP^{Sc} in samples from mice as early as 21 to 49 days post-infection (d.p.i.). The rapid rise in brain PrP^{Sc} between 49 and 98 d.p.i. was detected by all five tests; moreover, all five tests reached saturation in the detection of PrP^{Sc} between 147 and 220 d.p.i.

Our results demonstrate that BSE-infected Tg4092 mouse brains can be used for the assessment of rapid BSE test performance and the quality control of rapid BSE tests, as well as for the standardization of secondary reference materials.

Results and discussion

Tg4092 mice were intracerebrally inoculated with a first-passage pool of homogenized brains of BSE-infected Tg4092 mice. Uninoculated control and BSE-infected Tg mice were housed under the same conditions and brains were harvested at specified time points. The brains were preserved in an argon atmosphere and did not show any

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signs of desiccation up to 28 months after collection. Measurements with the five rapid BSE tests (designated A–E) were performed between 7 months (samples 98 d.p.i.) and 14 months (samples 7 d.p.i.) after harvesting of the brains. Additional analyses on a subset of samples were performed with assay E approximately 12 months after the initial measurements, confirming the stability of the samples for at least 2 years (data not shown).

Bioassays were performed to monitor infectivity in brains of BSE-infected Tg4092 mice. For each of the six time points (7, 21, 49, 98, 147, and 220 d.p.i.), one hemisphere from 18 different mice were pooled and inoculated into eight Tg4092 mice. A total of 48 mice were infected with six different inocula. All mice developed clinical signs of disease after 233 ± 13 d.p.i. (pool 220 d.p.i.), 280 ± 48 d.p.i. (pool 147 d.p.i.), 302 ± 26 d.p.i. (pool 98 d.p.i.), 319 ± 36 d.p.i. (pool 49 d.p.i.), 367 ± 26 d.p.i. (pool 21 d.p.i.), and 478 ± 59 d.p.i. (pool 7 d.p.i.) (Figure 1).

Five rapid BSE tests (Bio-Rad TeSeE[®], Prionics Check[®] LIA, Fujirebio Frelisa[®], Idexx HerdChek[®], AJ Roboscreen[™] BetaPrion) were used to analyze 24 brain samples from Tg4092 mice at each of the incubation times (7, 21, 49, 98, 147 and 220 d.p.i.) for a total of 144 samples. In addition, each test was used to analyze 12 uninfected control samples. Each sample was measured in duplicate. All assays were able to process and analyze brain tissue from Tg4092 mice, although these tests were developed and optimized for diagnosis of bovine tissues. The low average weight of mouse brain hemispheres of approximately 200 ± 70 mg and the usual wet sample

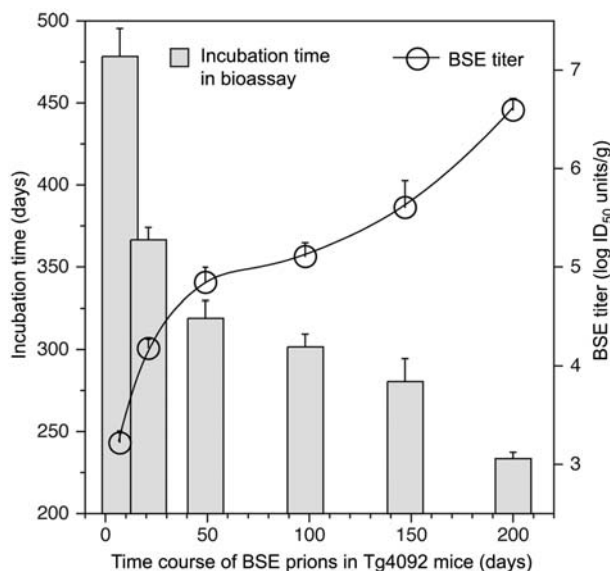


Figure 1 Time course of BSE prion replication in Tg(BoPrP^{+/+}) Prnp^{0/0}/4092 mice.

Tg4092 mice were inoculated intracerebrally with 1% brain homogenate from a BSE-infected cow; mice were euthanized at the indicated time-points post-inoculation and the brains were assayed for BSE prion titer in Tg4092 mice by the incubation time assay (Prusiner et al., 2004). The BSE prion titer was calculated from the incubation time and a calibration curve constructed previously (Safar et al., 2002). Data points are mean \pm SEM calculated from 8–12 brains assayed for each time point of the BSE time course (7, 21, 49, 98, 147, and 220 d.p.i.).

intake of between 200 mg (Fujirebio Frelisa) and 500 mg (Prionics Check[®] LIA) required adjustment of the amount of homogenization buffer to achieve an optimal tissue/buffer ratio for each of the tests. Duplicate measurement of homogenates (data not shown) indicated sufficient homogenization of samples.

Four of the five BSE tests use different concentrations of proteinase K (PK) to digest residual cellular PrP (PrP^C) in the homogenate. This purification step for PrP^{Sc} is crucial and had been optimized for bovine brain tissue. A 10.4 ± 2.1 -fold overexpression of bovine PrP^C in the brains of Tg4092 mice, compared to MoPrP^C in the parental FVB mice, resulted in a relative increase in residual BoPrP^C in digested homogenate compared to bovine-derived samples. This resulted in a baseline shift and elevated readings of uninoculated control samples (tests A–C) when analyzed with two of the four assays using limited amounts of PK. While PK is not used in a third test, another protease (a combination of trypsin and nucleases) is employed by this assay (Figures 2 and 3). The PK used in tests D and E digested Tg4092 mouse brain homogenates to the same extent as homogenates of BSE-negative bovine brainstems (Figures 2 and 3).

This baseline shift required the introduction of a provisional ‘transgenic mouse brain-specific’ cutoff for all tests to determine the initial detection of *de novo*-formed PrP^{Sc}. These cutoffs were calculated from the average reading of uninfected control samples plus three standard deviations. Using this ‘transgenic mouse-specific’ cutoff, we demonstrated that most tests begin to detect *de novo* prion synthesis between 21 and 49 d.p.i. Statistical analysis of variance (ANOVA) of results from all time points compared to the mean of readings of controls showed a significant increase in OD values from 49 d.p.i. (tests A, B, C) and 98 d.p.i. (tests D, E) onwards (data not shown). Considering the bioassay data and the inverse exponential relationship between titers of BSE prions, infectivity from the 49-d.p.i. inoculum corresponds to a titer of 4.9 log ID₅₀ and the 98-d.p.i. inoculum to 5.1 log ID₅₀/g brain tissue (Figure 1; Safar et al., 2002), indicating an approximately two-fold increase in prion load between those two time points.

All mice of the 220-day group developed clinical signs of disease with a mean incubation period of 201 d.p.i., with the first animal developing clinical signs at 188 d.p.i. and the last animal at 217 d.p.i. A small proportion of the samples from the 49-, 98-, and 147-d.p.i. groups was classified as negative with the various tests. To identify the reasons for these negative test results, the second hemispheres of the negative brains were tested with another assay. Such infrequent negatives can be explained either by biological variation at these early time points, or less likely, by a failure of the assay in question. The samples affected were excluded from the analysis. Examination of the genealogy of the Tg4092 mice gave no indication that any of the excluded samples were derived from a group of mice more closely related than the other Tg mice. All of the Tg4092 mice used in this study were controlled for stable integration of the bovine PRNP transgene.

All uninfected control samples analyzed with test A had elevated OD_{450/620 nm} readings compared to both BSE-

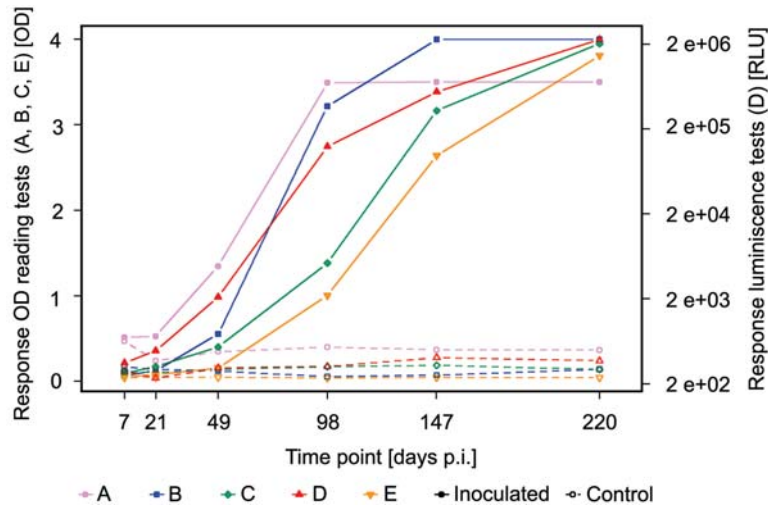


Figure 2 Detection of prions in infected mouse brains by five assays. Median of results obtained for up to 24 different mouse brains per time point of infection for each of the five assays. The y-axis corresponds to OD values for tests A, B, C and E, or to light units recorded for test D. The continuous lines connect the median values of inoculated brains; the dashed lines connect the median values of uninoculated control brains.

negative bovine samples and mouse brain tissues exhibiting a normal level of mouse PrP expression (data not shown). Eleven of 12 control samples were above the usual bovine cutoff of this assay. A second laboratory analyzing a second subset of 156 Tg4092 samples with test A obtained the same results. By applying a transgenic mouse brain-specific provisional cutoff (0.674), all control samples could be classified negative. Using this approach, 22 of 24 samples of the 49-day group were scored as positive (Figure 3A).

Test B applies a dynamic cutoff for the classification of bovine samples. Here, only one of 12 control samples was above the cutoff ($OD_{450/620\text{ nm}}=0.188$, cutoff 0.173), but the majority of control samples had elevated $OD_{450/620\text{ nm}}$ values compared to BSE-negative bovine samples. A cutoff shift from approximately 0.18 to 0.259 (control sample mean+3SD) led to a more stringent separation of the negative and positive population of data from Tg4092 mouse brains. Using this shifted cutoff value, only two of 22 samples of the 49-day group were

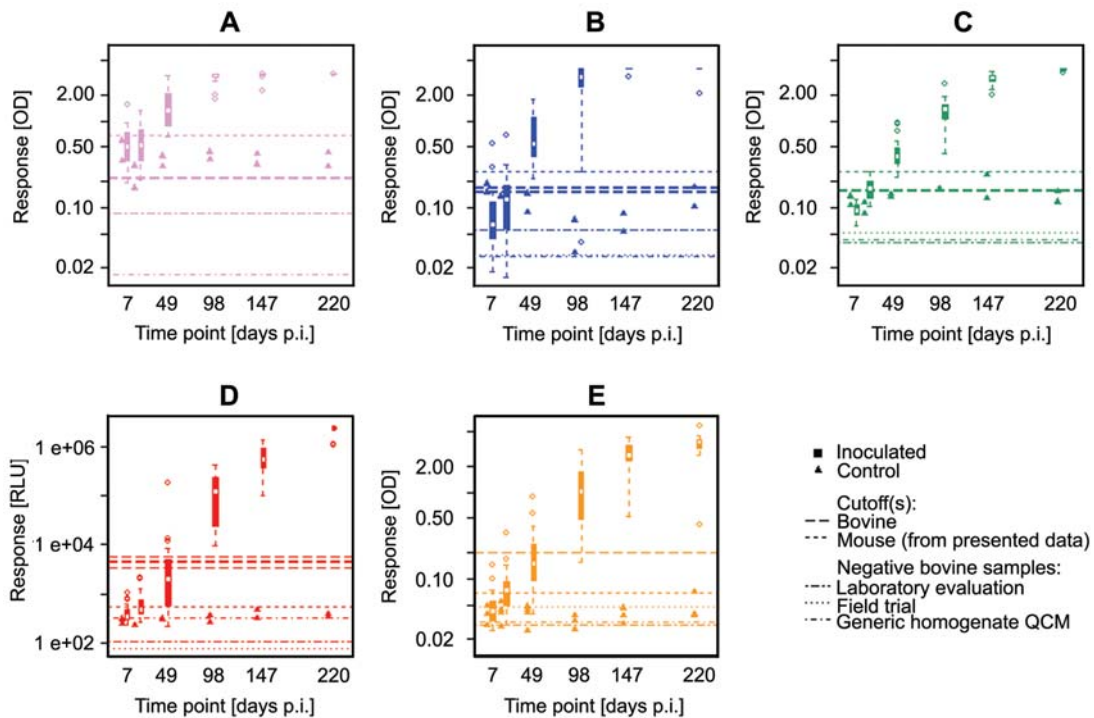


Figure 3 Box-whisker plots (A–E) of results obtained with tests A–E, respectively. The triangles represent uninoculated control samples. The different formats of dashed lines represent the normal ‘bovine’ cutoffs and provisional ‘transgenic mouse-specific’ cutoffs, as well as the mean values obtained with bovine nervous tissue samples and the IRMM QCM homogenate. The time points of infection are given on the x-axis; the response is given on the y-axis. Statistical outliers are indicated by circles.

classified as negative, whereas no samples from this group were classified as negative with the common cut-off calculation. The above two samples were excluded from this analysis, since the other hemispheres were classified as negative by test E.

Using the cutoff for bovine brains (0.159) in test C, one control sample was classified as positive (0.239) and two of 22 samples from the 49-day group were classified as negative. Shifting the cutoff to 0.259 (control mean + 3SD), no control samples were positive and four of 22 samples of the 49-day group were negative. Comparing the median OD_{450/620 nm} value for the control samples (0.133) and that for negative bovine samples (0.051) with test C lends further support to the utility of modifying the cutoff value for Tg4092 brains that have elevated levels of BoPrP^c.

Tests D and E digested BoPrP^c in Tg4092 samples to the same extent that BoPrP^c was hydrolyzed in bovine samples. Applying the bovine cutoff for both tests, BoPrP^{sc} was detected between 49 and 98 d.p.i.; the use of a modified and, in this case, lower transgenic mouse-specific cutoff resulted in BoPrP^{sc} detection from samples at 49 d.p.i. for both tests.

The variation among brains from one experimental group was similar to that found for other groups of brains collected from other time points. The extent of variation in the data evident in Figure 3 is mainly dependent on biological effects such as the rate of PrP^{sc} propagation during the incubation periods between 21 and 147 days. Some of the apparent variation in samples may be due to intrinsic fluctuations in robustness and repeatability of rapid BSE tests. Since the Tg4092 mice were inoculated in small groups as they were produced, we questioned whether this protocol was responsible for variations observed with one particular rapid BSE test. Variations in samples measured by one specific test could not be confirmed by one or more of the other tests. This finding argues that the Tg mice need not be inoculated at the same time.

Our studies demonstrate that BSE-infected Tg4092 mouse brains can be used as a 'general standard' for performance assessment and quality control of regulated rapid post mortem BSE tests. Our studies demonstrate that five different rapid BSE tests are able to detect BoPrP^{sc} in Tg mouse brains, which fulfill the requirements for a reliable reference material, i.e., homogeneous and stable. The study design with Tg4092 mice mimics, in a controllable and repeatable way, a natural 'dilution series' with bovine-derived prions, the target of rapid post mortem BSE tests. It is generally thought that Tg mice reproduce most of the characteristics of natural prion infections in humans, livestock and cervids, and that passage of a particular prion strain from a natural host to Tg mice often preserves the characteristics of the original prion (Scott et al., 1989; Asante et al., 2002; Safar et al., 2002; Korth et al., 2003; Supattapone et al., 1999; Scott et al., 2005).

From the results presented here we conclude that Tg mouse brains offer many important advantages as reference materials compared to nervous tissue from large animals such as cattle (Table 1). EU-approved rapid BSE tests require periodic quality control, which must be performed with calibrated reference materials. BSE-infected Tg4092 mouse brains are a renewable and economically feasible source of reference material that can be employed both to assess the quality and capabilities of already approved tests and to evaluate new tests.

Materials and methods

Transgenic mice

The Tg(BoPrP^{+/+})4092/*Pmp*^{0/0} mice were developed by InPro Biotechnology (South San Francisco, CA, USA). These mice express bovine PrP, as described elsewhere (Scott et al., 1997, 1999).

Table 1 Comparison of BSE-infected bovine brainstem and BSE-infected Tg4092 mouse brains as reference samples.

Characteristic	Tg mouse brains	Bovine brainstem
Heterogeneity of PrP ^{sc} distribution	Negligible, use of brain hemisphere, usually symmetric distribution	Very high, important to exclude intact bovine tissue samples for use as standard
Homogenization	Intact tissue, no sub-sampling	Tissue or homogenate sub-sampling from tissue necessary, adding random effects
Dilution series	Natural, depending on d.p.i. at harvest	Heterogeneity, homogenization needed
Availability	Not limited	Very limited
Variation in PrP ^{sc} concentration between individuals	Can be monitored	Problematic, would require whole brainstem for assessment
PrP ^{sc} concentration	Consider between samples	Between and within samples
Renewable	Yes	Each sample unique
Analytical sensitivity of diagnostic tests	Comparable	Difficult due to heterogeneity
Species barrier for bovine prions	No	No
Costs	High	High
Potential as standard	Yes	No, due to heterogeneity and low sample intake; only as homogenate related to a stable reference material

Inoculation with BSE prions

Inoculations were performed intracerebrally with a defined standard aliquot (30 μ l) of a first-passage homogenate of 20 pooled BSE-infected Tg4092 mouse brains, originally inoculated with a 10% (w/v) homogenate prepared from the medulla of a BSE-positive Hereford bull (PG31/90). Dilutions of 10% and 1% of the inoculum were analyzed with the InPro CDI rapid *post mortem* BSE test, following the protocol originally applied in the EU evaluation. The inoculum had a mean endpoint titer of $10^{6.9}$ ID₅₀ units/g of brain. A 1% (w/v) brain homogenate in PBS was used and additional aliquots were stored at -80°C for future inoculations. A total of 798 mice were inoculated; 133 mice served as an uninoculated control group. The Tg mice were divided into three experimental groups per time point, with inoculation of each Tg mouse group performed on two or three different dates.

Collection of samples

Harvested brains were split into two hemispheres and immediately frozen on dry ice, distributed into pre-labeled, argon-flushed, 1.8-ml cryo-tubes (Nunc, Rochester, NY, USA) and stored at -80°C . Mice sacrificed at approximately 220 d.p.i. exhibited clinical signs of prion disease (Carlson et al., 1994).

Presence of transgene

Tail biopsies of all mice included were taken at weaning, DNA was extracted, Southern blotted, and probed for the transgene with a SHa 3'UT sequence. All animals with stable integration of the transgene were kept in the study.

Control expression level

To determine the level of PrP expression, 14 different mouse brains were homogenized in PBS. The protein concentration was determined by BCA assay and the concentration adjusted to 0.1, 0.05 and 0.025 mg/ml. The brain homogenates were then denatured in SDS sample buffer by boiling and subjected to SDS-PAGE. The gels were blotted onto a PVDF membrane, probed with a monoclonal anti-PrP antibody, detected using ECL, and visualized with X-ray film. The band intensities of the Tg4092 samples, wild-type FVB samples, and a *Prnp*^{0/0} control were quantified densitometrically, and the expression levels of Tg4092 samples were expressed relative to FVB mice.

Bioassay

One hemisphere from 18 different mouse brains from each time-point were pooled, homogenized, and used to inoculate 8 Tg4092 mice, resulting in a total of 48 mice for six time-points. These mice were observed for the onset of clinical symptoms and were confirmed to be BSE-positive after death.

Selection of and analysis with rapid BSE assays

Selected BSE tests were EC-approved assays with good repeatability and a sufficiently wide dynamic range for the purpose of the study (Philipp et al., 2004) or tests with a high relative analytical sensitivity, as demonstrated in the IRMM 2002 and 2004 BSE test evaluation exercises (Philipp and Vodrazka, 2004). Brain hemispheres from 24 different inoculated mice per time point were analyzed. The average sample weight of approximately 0.2 g did not correspond to the minimal initial weight intake defined for several assays. For those tests used in this study, the volume of homogenization buffer was adjusted to achieve an optimal protein concentration in the homogenate. Analyses were performed as defined by the test developer.

Statistical analysis

Statistical analysis was performed with the R free software environment for statistical computing and graphics (www.r-project.org). All valid data points and means of duplicate readings were considered for analysis; a few selected samples were excluded from the analysis on the basis of biological evidence. Duplicate measurements were used to assess the repeatability of assays. ANOVA was applied to assess the influence of various parameters on the experiment.

Acknowledgments

We would like to thank J. Safar, B. Oesch, J.-Y. Madec, T. Baron, J.-P. Bourgeois, E. Miyagawa, V. Leathers, M. Groschup, N. Kollmorgen and P. van Iwaarden for support and discussions. D.G., K.G. and S.B.P. have financial interests in InPro Biotechnology, Inc.

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Received September 19, 2006; accepted October 6, 2006