



Research report

PrP-C1 fragment in cattle brains reveals features of the transmissible spongiform encephalopathy associated PrP^{Sc}

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ABSTRACT

Three different types of bovine spongiform encephalopathy (BSE) are known and supposedly caused by distinct prion strains: the classical (C-) BSE type that was typically found during the BSE epidemic, and two relatively rare atypical BSE types, termed H-BSE and L-BSE. The three BSE types differ in the molecular phenotype of the disease associated prion protein, namely the N-terminally truncated proteinase K (PK) resistant prion protein fragment (PrP^{res}). In this study, we report and analyze yet another PrP^{res} type (PrP^{res-2011}), which was found in severely autolytic brain samples of two cows in the framework of disease surveillance in Switzerland in 2011. Analysis of brain tissues from these animals by PK titration and PK inhibitor assays ruled out the process of autolysis as the cause for the aberrant PrP^{res} profile. Immunochemical characterization of the PrP fragments present in the 2011 cases by epitope mapping indicated that PrP^{res-2011} corresponds in its primary sequence to the physiologically occurring PrP-C1 fragment. However, high speed centrifugation, sucrose gradient assay and NaPTA precipitation revealed biochemical similarities between PrP^{res-2011} and the disease-associated prion protein found in BSE affected cattle in terms of detergent insolubility, PK resistance and PrP aggregation. Although it remains to be established whether PrP^{res-2011} is associated with a transmissible disease, our results point out the need of further research on the role the PrP-C1 aggregation and misfolding in health and disease.

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1. Introduction

Transmissible Spongiform Encephalopathies are a group of neurodegenerative diseases which share a particular etiologic agent consistent with an infectious protein, termed Prion (Prusiner et al., 1981; Prusiner, 1982). Prions result from a conformational switch of the normal host-encoded prion protein (PrP^C) to a beta-sheet enriched disease-associated isoform (PrP^{Sc}). PrP^{Sc} is able to accumulate within the CNS owing to newly acquired biological and physico-chemical properties such as insolubility in non-

denaturing detergents, propensity to aggregate and relative resistance to protease digestion. Routine diagnostic tests rely on these properties for identification of PrP^{Sc} in the form of the PK-resistant core fragment (PrP^{res}) (Silva et al. 2015).

Bovine spongiform encephalopathy (BSE) is a prion disease of cattle which reached epidemic proportions in the mid-1980s and was later causally linked to the occurrence of a new variant of Creutzfeldt-Jakob Disease (vCJD) in humans (Will et al., 1996; Collinge et al., 1996; Hill et al., 1997). Monitoring of the extent of the BSE epidemic and of the efficacy of the control measures to stop its spread among cattle and to humans was done through active disease surveillance (Ducrot et al., 2008). Active surveillance relies on the screening of cattle through a first line of rapid tests, primarily enzyme-linked immunosorbent assay (ELISA)-based, which are followed when needed by confirmatory tests, mainly immunohistochemistry (IHC) or Western blot.

Three BSE strains, namely C-BSE, H-BSE and L-BSE, have been identified on the basis of the different biochemical properties of

Abbreviations: TSE, transmissible spongiform encephalopathy; BSE, bovine spongiform encephalopathy; PK, proteinase K; PrP, prion protein; PrP^{res}, protease-resistant prion protein; PrP^{Sc}, disease-associated PrP; PrP^C, normal cellular PrP; NaPTA, sodium phosphotungstic acid; RT-QuIC, real time quaking induced conversion.

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the PrP^{res} fragments, and on their different phenotypes induced upon experimental transmission (Casalone et al., 2004; Biacabe et al., 2004). C-BSE is the strain that was typically associated with the epidemic, whereas H-BSE and L-BSE are two rarely occurring conditions that were identified in old cows. H-BSE and L-BSE are caused by atypical prion strains. They may pose a risk to humans as they are hypothesized to be a possible origin for the C-BSE epidemic, and L-BSE in particular has been shown to induce prion disease experimentally in non-human primates (Beringue et al., 2007; Torres et al., 2011; Bencsik et al., 2013; Mestre-Frances et al., 2012). In 2011, as part of the disease surveillance in Switzerland, two relatively old cows (case 1: 8 years and case 2: 15 years of age) with an unclear clinical history were tested positive in both rapid and Western Blot confirmatory assays, and revealed an unusual PrP^{res} (PrP^{res-2011}). PrP^{res-2011} was N-terminally truncated and of lower molecular mass when compared to those associated with the known BSE types. This finding raised the hypothesis of the presence of a novel BSE prion strain (Seuberlich et al., 2012). Yet, further investigation for disease specific brain lesions and immunohistochemistry were hampered by the severely autolytic state of the brainstems. Field samples commonly exhibit various degrees of autolysis especially during summer time, and PrP^{res} fragments similar to PrP^{res-2011} have been described in the literature, sometimes associated with autolysis (Carra et al., 2009; Dudas et al., 2015). Another possibility would thus be that PrP^{res-2011} is a physiologically occurring fragment that was improperly digested, perhaps due to advanced tissue autolysis (Kittelberger, 2012). However, extensive investigation of such unusual molecular PrP^{res} phenotypes is still lacking.

While bioassays have been initiated to address the question of infectivity and the presence of a known or potentially new prion strain, a comparison of physico-chemical properties of PrP^{sc} and PrP^{res-2011} remained to be done.

In this study, we hypothesized that the PrP^{res-2011} resulted from particular physicochemical properties of the prion protein found in the 2011 cases, which differed from those of the normal PrP^c.

We first investigated the effects of autolysis on the PK activity and in the generation of PrP^{res} fragments, and we characterized the prion protein fragments present in the Swiss 2011 cases for comparison with PrP^c and with BSE associated PrP^{sc}. Our results confirm that PrP^{res-2011} corresponds in its primary structure to the truncated physiological PrP-C1, but at the same time reveals features, which are reminiscent of PrP^{sc}. These results raise fundamental questions about the role of PrP-C1 in the pathogenesis of degenerative CNS disorders.

2. Results

2.1. PrP-C1 is the predominant prion protein species in BSE negative autolytic field samples and in the 2011 cases

In order to identify PrP fragments present in autolytic and fresh BSE negative and in BSE positive brain samples, we used a set of monoclonal antibodies that bind to defined regions of the bovine prion protein (Fig. 1A). In fresh BSE negative tissue samples as well as in a BSE positive control sample, three PrP species could be identified which revealed antibody binding patterns consistent with full-length (FL) PrP (26 kDa), PrP-C2 (18 kDa) and PrP-C1 (16 kDa) (Fig. 1B). Interestingly in the BSE negative samples, PrP-C2, which is considered to represent PrP^{res} in C-BSE, was observed in small amounts. In addition a small fragment of ~10 kDa was found with the N-terminal SAF32 antibody in some of the fresh brain samples, which supposedly corresponds to the PrP-N1 fragment. In autolyzed tissues of BSE negative cattle and of case 1 and 2, PrP-C1 was the main fragment found while PrP-C2 as well

as PrP-FL were almost entirely depleted (Fig. 1C and Supplementary Fig. 1). A similar effect was observed under laboratory conditions, when fresh BSE negative brainstem samples were incubated for up to 7 days at 36 °C and underwent severe autolysis (Supplementary Fig. 2). However, none of the autolytic BSE negative samples had detectable PK-resistant prion protein (data not shown). These data indicate that PrP-C1 is more resistant to autolysis than the other PrP species in healthy animals and that test reactivity in the 2011 cases is likely related to PrP-C1.

2.2. The PrP^{res-2011} fragment corresponds to PrP-C1

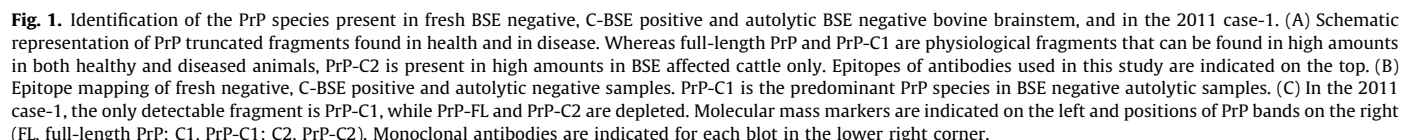
Next we aimed at investigating the PK sensitivity of the PrP-C1 in fresh and autolyzed BSE negative brainstem samples and in the brainstem tissue of case 1. Samples were incubated with different amounts of PK using the standard diagnostic Western blot test. In fresh tissue samples, a three-band pattern occurred at around 25 kDa, 20 kDa, and 16 kDa, when the PK was at concentrations below 2 µg/ml. This pattern was identical to that of PrP^{res-2011} digested with the standard test PK concentration of 100 µg/ml (Fig. 2A). Deglycosylation after PK treatment, showed that all three PrP species were readily identified in non- or mildly digested fresh negative samples. Yet, PrP-C1 was clearly more resistant to PK than PrP-C2 and PrP-FL in these samples (Fig. 2B). The PK resistance of PrP-C1 was similar in autolytic BSE negative samples (Fig. 2C, left panel), but much higher in the case 1 sample in this setup (Fig. 2C, right panel) when compared with that of the fresh or autolytic negative tissue. We have shown previously that PrP^{res-2011} in case 1 and case 2 have the same antibody binding profile as PrP-C1 in the present study and in both cases PrP^{res-2011} showed a similar increased PK resistance (Seuberlich et al., 2012)(Fig. 1). Taken together these results strongly support that PrP^{res-2011} corresponds in its primary sequence to PrP-C1, but with a higher protease resistance than PrP-C1 in BSE negative animals, and is hereafter termed PrP-C1^{res}.

2.3. PrP-C1^{res} is precipitated by sodium phosphotungstic acid (NaPTA)

NaPTA has been shown to selectively precipitate PrP^{sc} (Safar et al., 1998; Wadsworth et al. 2001), which has been proven useful for efficiently recovering and enriching PrP^{sc} from autolytic tissues (Huang et al., 2011). In order to identify possible scant amounts of PrP-C1^{res} in BSE negative autolytic field samples when using the standard test conditions, PK digestion was followed by NaPTA precipitation. Immunoblotting revealed that the PrP-C1^{res} was present in the NaPTA pellet fraction for case 1, but not in PK digested negative field samples, regardless of tissue condition (Fig. 3).

2.4. The PK activity is not inhibited in the Swiss 2011 brainstem samples

To investigate whether the detection of PrP-C1^{res} is the result of PK inhibition, we measured the PK activity in the digestion reaction of case 1 by an azocasein-based assay and compared it to that in fresh and in autolytic BSE negative samples. The linear range of the assay was first determined and found optimal when PK was used at a concentration of 25 µg/ml and the reaction was run for 10 min (Supplementary Fig. 3). The absorbance levels in the blank controls, i. e. in brain homogenates without PK, were at background levels. PK activity did not differ between the three sample matrices (Fig. 4). These findings do not support a role of PK inhibition effects in autolytic or in the 2011 swiss samples.



In contrast to PrP^C the TSE associated PrP^{Sc} is insoluble in non-denaturing detergent and recovered in the pellet following ultracentrifugation at 115,000xg for 1 h at 4 °C. We analyzed the solubility of the prion protein species found in case 1 and case 2 by ultracentrifugation and PK digestion. Similar to PrP^{res} in the C-BSE control, the PrP-C1^{res} was only detected in the insoluble fraction (P2) and not in the soluble one (S2) of case 1. Noteworthy, a similar signal was observed in the PK digested P2 fraction of a fresh sample of the cerebral hemisphere of case 2 (Fig. 5A). In both cases the insoluble PrP-C1^{res} was consistently found in three indepen-

dent analyses, and represented a small proportion of the total PrP-C1. Several BSE negative brain samples, from brainstem and cerebral hemispheres and from cows of different ages, were used as negative controls. In one sample of an older cow (age 8.5 years), a faint single band signal was present in the PK digested P2 fraction and had a molecular mass similar to the diglycosylated band of the PrP-C1^{res} (Fig. 5A). However, this was not the case in samples of cattle younger than 5 years (Supplementary Fig. 4). When PK digestion was followed by NaPTA precipitation, the detergent-insoluble PrP-C1^{res} was recovered in the NaPTA pellet, similar to the PrP^{res} from BSE affected cows (Fig. 5B).

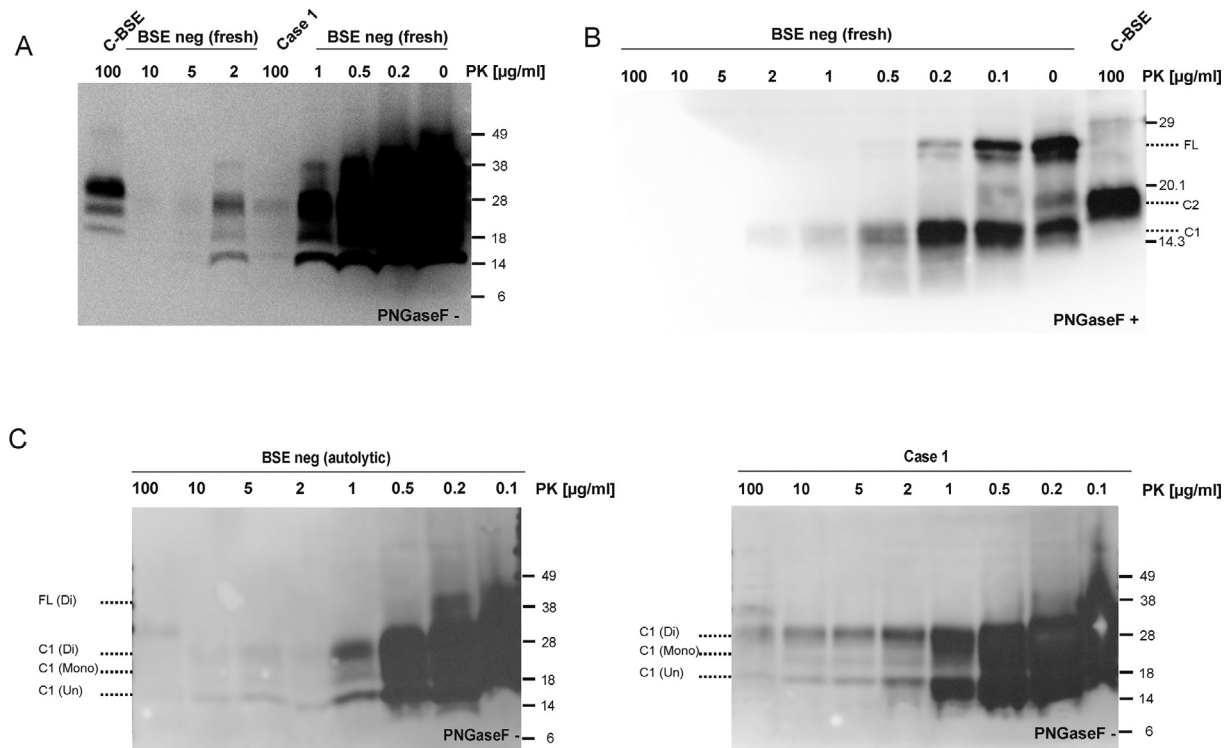


Fig. 2. PrP-C1 is more resistant to PK digestion in the Swiss 2011 case 1 than in fresh and autolytic BSE negative brain samples. (A) Fresh BSE negative brain samples were treated with different concentrations of PK and analyzed by Western blot. At PK concentrations ≤ 2 µg/ml glycosylated PrP-C1 is detected and reveals a similar molecular mass and banding pattern as in PrP^{res-2011} in case 1 under standard PK concentration. (B) Deglycosylation with PNGase F confirms that the presence of PrP-FL, PrP-C2 and PrP-C1. PrP-C1 is more resistant to PK than PrP-C2 and PrP-FL. (C) In the Swiss 2011 case 1 PrP-C1 is more resistant to PK than in autolytic BSE negative brain cattle tissue. Where possible, the glycoforms of PrP- and PrP-C1 are indicated: diglycosylated (di), monoglycosylated (mono) and unglycosylated (un).

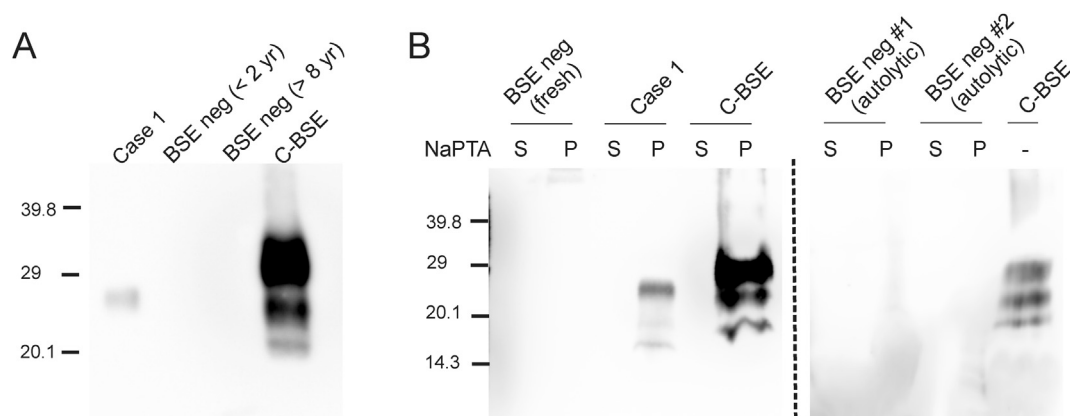


Fig. 3. PrP-C1^{res} is precipitated by NaPTA. (A) Western blot of brain tissue of the 2011 case-1 without NaPTA precipitation yields in some runs a faint band that might correspond to diglycosylated PrP-C1. BSE negative brain tissues (BSE neg) of a young and an aged bovine were included as controls. (B) When PK treated brain homogenate was NaPTA precipitated, PrP-C1^{res} was recovered in the pellet (P) of case 1 and was not detected in the supernatant (S). PrP-C1^{res} was not detected in BSE negative autolytic brain tissues.

2.6. PrP-C1^{res} consists of small oligomers

Velocity sedimentation is commonly used to separate proteins according to their molecular sizes and shape. In this study, we used this assay to identify sub-classes of PrP which exhibited PK-resistance in fresh negative and positive cases, and in the 2011 cases, and to analyze the distribution of aggregates within each type of samples. We found that both case 1 (autolytic brainstem tissue) and case 2 (fresh, cerebral hemisphere tissue) contained low to moderate amounts of PrP aggregates in the fractions with higher and intermediate sucrose concentrations (fractions 1 to 8),

which implies the presence of some PrP aggregates with sizes larger than expected (Fig. 6). Noteworthy, similar aggregates were also found in the negative control sample of the old cow, but were present in lesser amounts in younger cows (Supplementary Fig. 5). Yet, in all samples except the C-BSE control, most of the PrP localized in the upper fractions 10 to 12 with low sucrose concentration. Interestingly, after PK treatment, PrP-C1^{res} was recovered in these upper (low sucrose concentration) fractions in both the autolytic brainstem tissues of case 1 and the non-autolytic 2011 case-2, which indicates PrP-C1^{res} aggregates of small size.

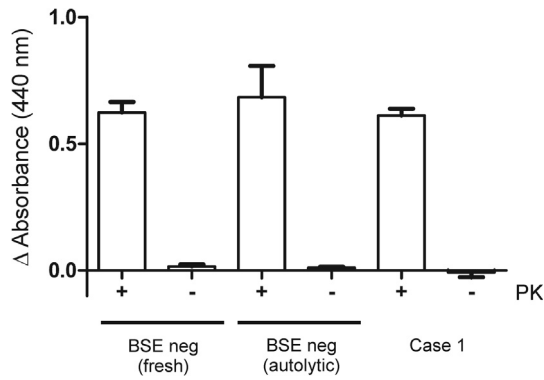


Fig. 4. PK activity is not inhibited in the Swiss 2011 case 1 brain tissue. Samples were analyzed by a fixed-time azocasein assay in four independent replicate runs. Bars indicate the SD.

2.7. PrP-C1^{res} has no seeding activity in vitro

To investigate whether PrP-C1 of the Swiss 2011 cases induces conversion of recombinant bovine PrP *in vitro*, homogenates of autolytic brainstem samples of case 1 and 2 were tested by the RT-QuIC assay. Results show that conversion times in both cases were on average slightly shorter compared to negative control tissues (Fig. 7). Though the Swiss case ThT-signal times differed from the BSE negative control values ($p < 0.001$, $d = 1.25$), the difference is far less as compared to the difference between the pooled C-, H-, and L-type BSE positive times versus the BSE negative control values ($p < 0.001$, $d = 8.16$). Given the extremely large difference in effect between Swiss case ThT-times versus BSE negative times, as compared to the difference between BSE positive control times and BSE negative times, it cannot be excluded that the differences between Swiss case ThT-signal times and BSE negative control

times is due to confounder unrelated to BSE, and more related to the inoculum or seed-homogenate condition. For instance, bacterial contamination may have been an issue. In any case, the Swiss cases do not possess the same seeding-capacity as the BSE+ control cases.

3. Discussion

Aside from full-length PrP, two additional N-terminally truncated forms of the prion protein have been characterized in brains, namely the PrP-C1 fragment of around 16 kDa, and the PrP-C2 fragment of around 18 kDa (Chen et al., 1995). The PrP-C1 fragment is found under physiological conditions and results from the N-terminal cleavage of the full-length PrP^c by endogenous cellular proteases (Vincent et al., 2001). The hallmark of prion-diseases is the presence of high amounts of the protease-resistant and detergent insoluble PrP-C2 fragment in affected brains or cell cultures. Both in this study and in other studies, this PrP-C2 is shown to occur physiologically in small amounts in healthy brain samples (Mange et al., 2004; Zhao et al., 2006). In our study, the expected PrP fragments were found in fresh BSE negative and in C-BSE positive brain samples upon deglycosylation and epitope mapping, whereas PrP-C1 was the main fragment recovered in autolytic samples and in the 2011 cases.

In active disease surveillance, samples from fallen stock cattle are submitted to the laboratories in a wide spectrum of degradation states. Although studies seem to agree that autolysis of BSE positive material does not impact on the detection of PrP^{sc} using the current available diagnostic tests (Race et al., 1994; Chaplin et al., 2002; Hayashi et al., 2004; Schaller et al., 1999), it appears that the testing results for BSE negative samples can be ambiguous when dealing with autolytic material (Carra et al., 2009; Meloni et al., 2010). Especially PrP^{res} fragments similar to PrP-C1 have been previously described in field samples and have been so far

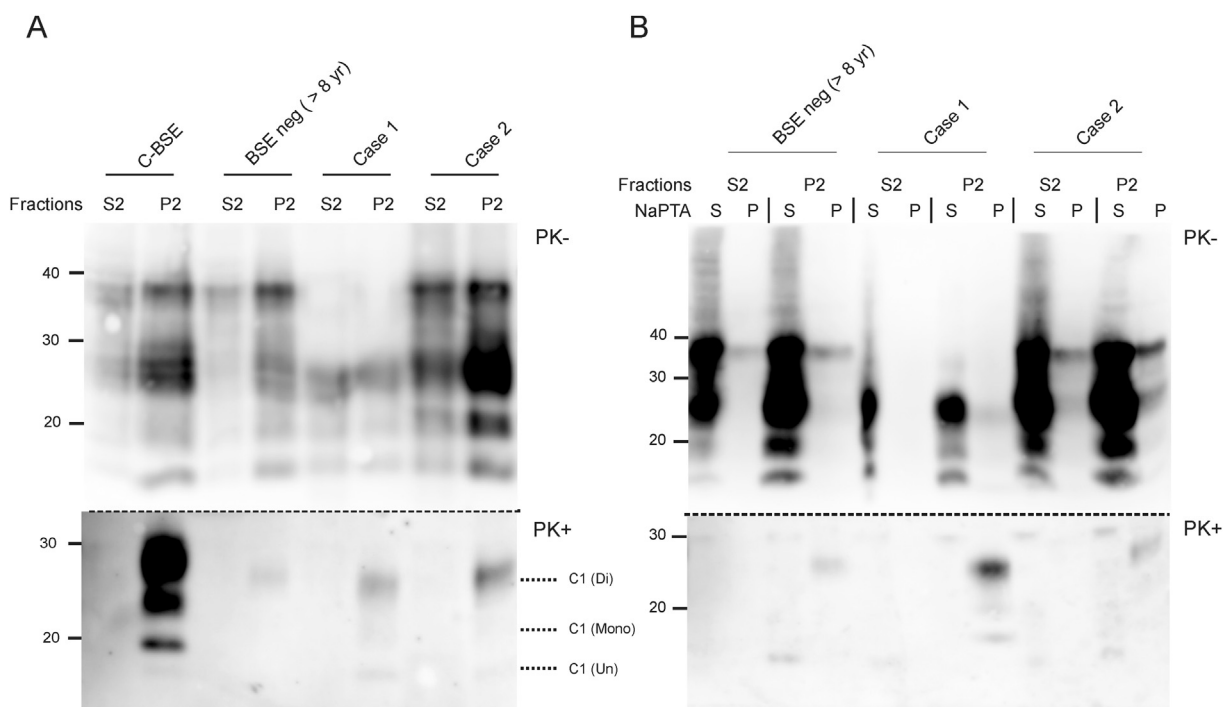


Fig. 5. Investigation of the soluble and insoluble PrP fractions present in the Swiss 2011 cases. (A) PrP-C1^{res} is detected in the insoluble (P2) fraction and not in the soluble (S2) fraction in the brain samples of the two Swiss 2011 cases (case 1, autolytic brainstem; case 2, non-autolytic cerebral hemisphere). In the brain sample of a BSE negative, old cow, a faint one-band signal was recovered in the insoluble fraction (P2) after PK digestion. No such fragment was found in the other negative brain samples tested. (B) The insoluble and PK-resistant PrP-C1^{res} is precipitated by NaPTA (S, supernatant; P, pellet).

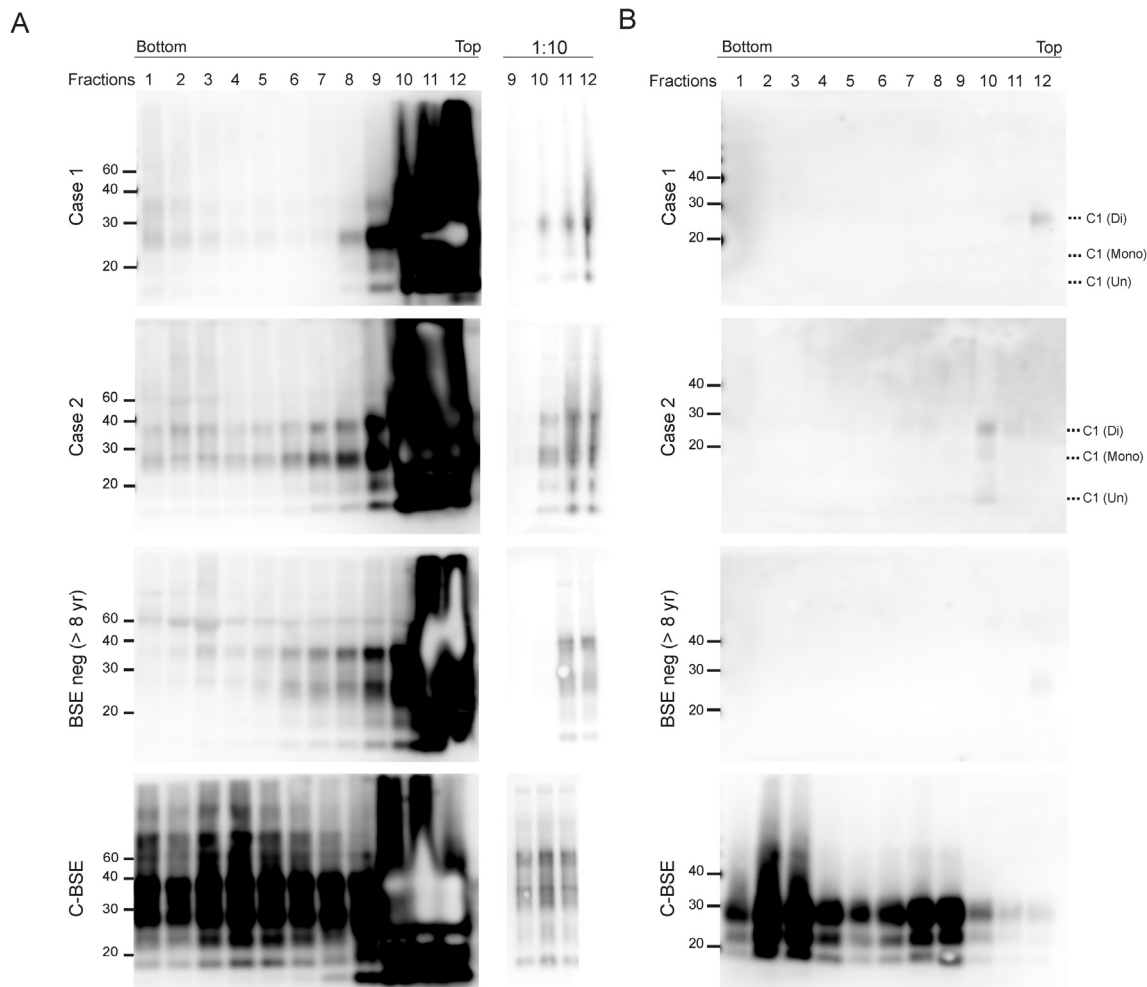


Fig. 6. Prion protein aggregation in brain tissues of the Swiss 2011 cases. (A) Brain homogenates were subjected to high-speed centrifugation through a sucrose-gradient ranging from 10% sucrose (fraction 12, top) to 45% sucrose (fraction 1, bottom). Individual fractions were analyzed for prion protein by Western blot. Both Swiss 2011 cases and the negative control of an aged cow revealed low amounts of aggregates with a wide range of sizes. Panel on the right corresponds to the fractions 9 to 12 diluted 1:10 before loading, in order to identify the bands present in these fractions. (B) The same fractions as in panel A were PK digested. Note the low amounts of PrP-C1^{res} in fraction 10–12 of case 2 and in fraction 12 of case 1. Brain tissue of a C-BSE case were included as controls.

considered a consequence of autolysis or improper PK digestion (Carra et al., 2009; Kittelberger, 2012; Dudas et al., 2015). In our study, we could not link tissue autolysis or PK inhibition to the recovery of protease-resistant PrP-C1 in the 2011 cases. First, similar PrP^{res} was found in no other autolytic field sample, even when applying NaPTA precipitation in an attempt to concentrate PrP^{res}, nor in samples that were subjected to experimental autolysis under laboratory conditions. Second, the same protease-resistant PrP-C1 was recovered in the fresh brain hemispheres that were available for one of the 2011 cases. And third, PK activity was detectable at similar levels in reactions using the sample matrix of case 1 when compared to autolytic and fresh control matrices, ruling out an effect of PK inhibitors or degradation of PK in the 2011 cases as the cause for this particular PrP^{res}. PrP^c conversion to PrP^{sc} has also been attributed to bacterial factors other than PK inhibitors (Dudas et al., 2015). Especially, bacterial lipopolysaccharide (LPS) has been shown to have the ability to mediate the conformational conversion of recombinant PrP (Saleem et al., 2014). In our study, preincubation of fresh negative samples with various amounts of LPS prior to experimental autolysis did not yield any detectable PrP^{res} (data not shown). Taken together, all this indicates that the recovery of PrP-C1 in the 2011 cases following PK treatment does not result from tissue autolysis and/or

reduced PK activity, and it rather reveals an intrinsic PK-resistance of this particular PrP fragment. Consequently, this partially PK resistant PrP-C1 has been termed PrP-C1^{res}.

The results of the detergent insolubility assay together with the NaPTA precipitation assays show that PrP-C1^{res} appears to share additional biochemical characteristics with PrP^{sc} in terms of insolubility, aggregation and PK resistance. Noteworthy is the variable intensity of the 2011-PrP^{res} signal and thus its amounts when using PK-digestion alone. However, it was consistently recovered when NaPTA precipitation was done following PK digestion. In the brain of one old non-affected cow, a small amount of detergent-insoluble PrP^{res} was found in the form of a single band signal upon immunoblotting, indicating the potential presence of insoluble PrP^{res} in the brain of aged cattle.

In order to further investigate the similarities between the PrP-C1^{res} and PrP^{sc}, and because the PrP^{sc} fraction of infected animals is known to be composed of a continuum of size aggregates (Morales et al., 2016), we analyzed the size distribution of the PrP species present in the 2011 cases and compared it to BSE negative and BSE positive samples. Despite the recovery of moderate amounts of PrP aggregates in the bottom and intermediate fractions of the 2011 cases upon velocity sedimentation in sucrose gradient, PrP-

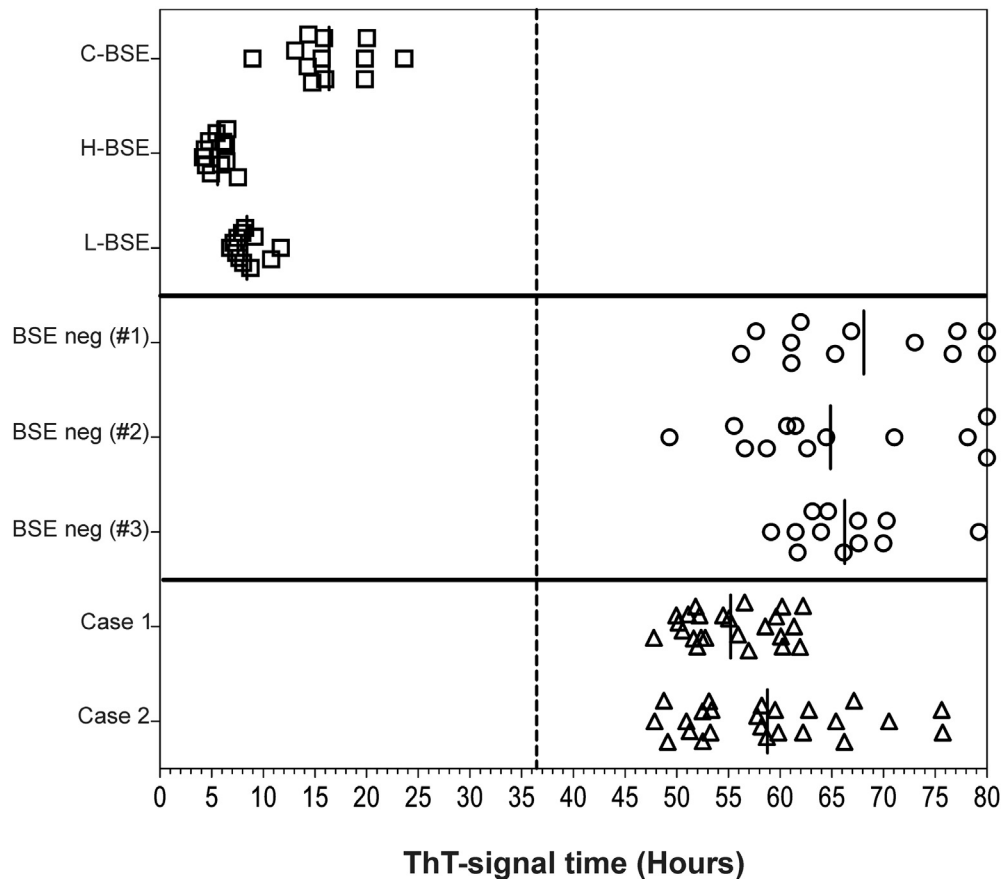


Fig. 7. Seeding activity of the Swiss 2011 tissue samples in RT-QulC. Brain homogenates from 3 known BSE positive (C-BSE, H-BSE and L-BSE), 3 known BSE negative (#1–#3) and the 2 Swiss 2011 cases were tested in 12 or more replicate wells using RT-QulC. This dot plot shows the times at which the individual replicate reaction fluorescence intensity crossed the threshold to indicate notable amyloid formation (ThT-signal time). All of the BSE positive reactions seeded amyloid formation well before the positive negative cutoff time of 36.46 h (dashed vertical line). Vertical continuous lines show median ThT-signal times.

C1^{res} was found in the upper fraction and thus correspond to small size PrP oligomers.

Small oligomers/low density aggregates of pathogenic prion protein have been found to have a high seeding potency, to be highly contagious and to determine the duration of the incubation time during transmission experiments (Kim et al., 2012; Tixador et al., 2010; Anaya et al., 2011). The RT-QulC assay, which mimics PrP seeding activity *in vitro*, was thus used to characterize the replicative abilities of the 2011 cases. Despite a statistically significant shift in the conversion rate of the 2011 strains when compared to the negative controls, the comparison with the positive controls showed that they do not possess a seeding capacity similar to known bovine prion disease. On another hand, not only does the RT-QulC response depend on the prion strain, but it also depends on the substrate (Orri et al., 2015; Masujin et al., 2016). Here, we used recombinant *E. coli* expressed full-length bovine PrP, which is neither glycosylated, nor processed to its physiological truncated forms, i.e. PrP-C1 and PrP-C2. RT-QulC reactions may therefore not reflect the *in vivo* situation. In this regard it may be interesting to establish an RT-QulC assay which uses glycosylated PrP-C1 as a substrate.

Collectively we found evidence that the unusual PrP^{res} observed in the two Swiss cattle brains represents small aggregates of PrP-C1 fragment with biochemical characteristics of PrP^{sc}, namely detergent-insolubility and PK-resistance. Pathological prions display a large strain variation, which is thought to ensue from the differential conformation adopted by the protein (Safar et al., 1998). This strain variation is often identified on the basis of the

biochemical differences of PrP^{res}, such as protease resistance and seeding potency, and on the occurrence of distinct phenotypic disease traits upon transmission. Other PrP conformers have been identified, which are referred to as silent prions because they are assumed to be present in normal brains in amounts that are too low to allow for the development of neurological diseases (Hall and Edsles, 2004). In particular, an N-terminally truncated, protease-resistant and detergent-insoluble PrP species termed insoluble PrP^c (iPrP^c) similar to the PrP-C1^{res} has been identified in the brains of uninfected humans (Yuan et al., 2006). One possibility is that iPrP^c is in fact an intermediate between PrP^c and PrP^{sc}. It has been proposed that silent prions are present in a reversible equilibrium with PrP^c, but that upon disruption of this equilibrium, they can accumulate within the brain tissue and lead to atypical forms of prion diseases (Biasini et al., 2008; Chiesa et al., 2008). Another interesting finding is that in humans affected with Alzheimer's disease, the aggregated A β protein interacts with iPrP^c which then seems to be pathologically implicated in the development of the disease. (Zou et al., 2011). Another concern is that the PrP-C1^{res} may be infectious and potentially reflects an early stage of a known or novel type of prion disease. However the question of infectivity of iPrP^c in humans could so far not be resolved. Bioassays in cattle have been conducted in parallel to this work in order to address the important question of infectivity of PrP-C1^{res} in cattle. Results will be reported soon and could potentially give insights into the role of PrP-C1^{res}/iPrP^c in disease pathogenesis in both cattle and humans.

Because the 2011 cases were old animals and protein aggregation is known to be part of the aging process (Labbadia and Morimoto, 2015), BSE negative animals of different ages were tested as negative controls in order to assess the potential role of aging in the appearance of protease-resistant PrP aggregates. It is important to note that in the old bovine brain with small amounts of insoluble PrP^{res}, as well as in the other negative control brains, a PrP^{res} signal was not found when using the PK conditions of the diagnostic tests, which is in accordance with previous studies that investigated brains of aged cattle for PrP^{res} (Tester et al., 2009). However, in the sucrose gradient experiment an increase in the amount of aggregates in the lower and intermediate fractions was noted in older animals when compared to young and middle-aged animals (Supplementary Fig. 3). Detergent-insoluble PrP species similar to PrP-C1 were also found in the brains of old-aged cattle, in higher amounts than in young animals, although a difference in PK resistance was not evidenced between young versus old animals (Yoshioka et al., 2010). These data point towards age-related differences in PrP aggregation in cattle. In view of the findings described here as well as in other studies, it appears that PrP-C1 may not only have a physiological function, but that it may also play a role in age-related neurodegenerative disorders both in humans and animals, which remains to be further investigated.

4. Experimental procedure

4.1. Tissue samples

From the two 2011 cases, only case 1 had enough brainstem material left to conduct the full panel of experiments. Other materials were not available for case 1. For the case 2, the amount of brainstem tissue was very limited. However, brain hemispheres samples were available, which had been kept for ~4 days at 4 °C post-mortem and which were morphologically still intact with only a mild degree of autolysis. Autolytic BSE negative cattle brainstem samples consisted of samples that were fully disintegrated (liquid stage) and that tested negative by an approved BSE screening test (Prionics Check PrioStrip). Fresh bovine brainstem tissues were collected from slaughtered calves. BSE-negative samples of brain hemispheres from old cows (age > 8 years) and middle-aged cows (around 5 years old), and BSE-positive brainstem samples were retrieved from the archives of the NeuroCenter, Vetsuisse Faculty, University of Berne, Switzerland. Approval by the local ethic committee was not required for this study. Laboratory procedures were approved according to the Swiss Ordinance on Handling Organisms in Contained Systems by the Federal Office for the Environment (Permission number A030162).

4.2. Sample preparation

Unless stated otherwise, samples were homogenized at 10% (w/v) in Prionics homogenization buffer with a FASTH device (Consul). All samples were analyzed with and without PK digestion and/or deglycosylation using standard methods. The PK digestion was done with the reagents of the Prionics Check Western kit, according to the manufacturer's instructions and at a final PK concentration of 100 µg/ml, unless stated otherwise. For PK titration experiments, the PK stock solution was diluted from 1:1 to 1:1000 in 1× homogenization buffer. Deglycosylation was performed for 90 min at 37 °C with PNGaseF (New England Biolabs) under denaturing conditions according to the manufacturer's instructions.

4.3. Western blot

Samples were denatured by boiling in SDS sample buffer and resolved on either 4–20% Tris-Glycine gradient gels (Life technologies) for epitope mapping, or on 12% Bis-Tris precast gels (Life technologies) for all the other experiments. Proteins were then transferred to PVDF membranes (Immobilon-P Millipore) for 1 hr at 150V. PrP probing was done by incubating the membrane with Sha31 antibody (0.1 µg/ml, overnight at 4 °C) as the primary antibody and a horseradish-peroxidase rabbit anti-mouse conjugate (Dako) as a secondary antibody, unless otherwise mentioned. Antibody binding was visualized with the ECL prime reagent kit (GE healthcare) and the LAS3000 chemiluminescence camera (Fuji). For epitope mapping, we used bovine PrP specific antibodies recognizing epitopes that span from the N-terminal to the C-terminal regions, namely SAF32 (Feraudet et al., 2005), 9A2 (Langeveld et al., 2006), Sha31 (Feraudet et al., 2005), and ROS-JB10 (McCutcheon et al., 2014).

4.4. Azocasein PK activity assay

Azocasein stock solution was prepared by dissolving azocasein (2% [w/v]) (Sigma-Aldrich) in Tris-Cl (100 mM, pH 8). Sample material consisted of brain homogenates (10% [w/v]) prepared in homogenization buffer (Prionics). For each reaction, 100 µl of tissue homogenate were mixed with 100 µl of azocasein stock solution, 10 µl PK stock solution and 10 µl digestion buffer of the Prionics Western Blot kit. Negative controls were prepared by omitting PK. After incubation at 48 °C for 40 min, 10 µl of stop solution (Prionics) was added. The remaining undigested azocasein was precipitated by an equal volume of trichloroacetic acid (15% [w/v]), followed by centrifugation at 15,000g at 4 °C for 10 min. One hundred µl of the supernatant was transferred to a microtiter plate and mixed with 100 µl of 1 M NaOH. The absorbance was read on a 96-well plate spectrophotometer at 440 nm (Versamax; Molecular Devices). In order to determine the optimal reaction conditions, reaction kinetics were first established with four different PK concentrations and using homogenizing buffer instead of the sample material. Once the appropriate amount of PK and incubation time determined, homogenates of fresh, autolytic and case 1 brain tissue were tested under these conditions in quadruplicate. The differences in absorbance between the fixed time point and t0 were compared between the samples.

4.5. Autolysis experiment

Fresh BSE negative brainstem samples were homogenized with an equal amount of distilled water and were incubated in a humid chamber at 36 °C for up to 7 days. Samples were collected on different days and analyzed by Western blot.

4.6. Separation of detergent-soluble and -insoluble PrP by one-step ultracentrifugation

Brain homogenates and detergent-soluble and -insoluble fractions were prepared as previously described (Yuan et al., 2006; Xiao et al., 2012). Briefly, 10% (w/v) brain homogenates were suspended in 9 volumes of lysis buffer (10 mM Tris, 150 mM NaCl, 0.5% Nonidet P40, 0.5% deoxycholate, 5 mM EDTA, pH7.4) on ice using a pestle. Five hundred µl of brain homogenate was then centrifuged at 1000g for 10 min at 4 °C and the supernatant was collected for ultracentrifugation at 115,000g (35,000 rpm) in a P55ST2 rotor and a Hitachi CP100NX centrifuge for 1 h at 4 °C. The supernatant S2 (detergent-soluble fraction) was transferred to a clean tube and the pellet P2 (detergent-insoluble fraction) was resuspended in 500 µl lysis buffer. Half of the volume of S2

and P2 were subjected to PK digestion with a final concentration of 50 µg PK/ml (Roche). The other half was left undigested. Following PK digestion, some samples were NaPTA precipitated (see below). In this case the resulting pellet was resuspended in 100 µl PBS with 0.1% (w/v) sarcosyl for the non-PK-digested samples, and in 20 µl PBS sarcosyl for the PK digested samples. Each sample was boiled at 100 °C for 5 min with an equal amount of SDS sample buffer and analyzed by Western blot.

4.7. Sodium phosphotungstic acid (NaPTA) precipitation

NaPTA precipitation was done on selected samples digested according to the Prionics Check Western kit, as well as on S2 and P2 fractions with and without PK digestion. Samples were mixed with an equal volume of PBS with 4% (w/v) sarcosyl and incubated at 37 °C for 10 min. Benzoinase (Merck, final concentration 50 units/ml) and MgCl₂ (final concentration 1 mM) were added to the samples followed by incubation at 37° for 1 h. NaPTA stock solution containing 4% (w/v) sarcosyl was then added to obtain a final concentration of 0.3% (w/v) NaPTA (Sigma-Aldrich). Samples were incubated at 37 °C for 30 min with constant shaking, and centrifuged at 15,000g for 30 min at 25 °C. Pellet and supernatant were separated and the supernatant's protein content was precipitated by adding 4 times the volume methanol, with subsequent incubation at -20 °C for 15 min and centrifugation at 10,000g for 10 min. Pellets were resuspended in PBS with 0.1% (w/v) sarcosyl.

4.8. Velocity sedimentation in sucrose step gradient

Brain homogenates (10% [w/v]) were prepared in PBS with 1% sarcosyl. The supernatant was recovered after pre-centrifugation at 1000g for 5 min and was loaded on top of a sucrose gradient, prepared by superimposing 350 µl layers of 45%, 40%, 35%, 30%, 25%, 20%, 15%, and 10% sucrose solutions from bottom to top. The gradients were centrifuged at 200,000g (46,000 rpm) for 65 min at 4 °C using a P55ST2 rotor and a Hitachi CP100NX centrifuge. After centrifugation, the contents of the centrifuge tubes were sequentially removed from the bottom to the top to collect 13 fractions of approximately 250 µl each. Half of each fraction was subjected to PK digestion (50 µg/ml) for 1 h; the second half remained undigested. The samples were subjected to Western blot analysis to detect PrP^C/PrP^{Sc} or PrP^{res} in the individual fractions.

4.9. Real time quaking induced conversion (RT-QuIC) assay

Samples were homogenized to 10% w/v in homogenization buffer (PBS, 0.15 M NaCl, 1% NDSB 201, 0.1% SDS, 1 mM EDTA and 1 × protease inhibitor [EDTA free complete protease inhibitor cocktail, Roche]) and were diluted to 0.1% (w/v) homogenate in seed diluent (PBS, 0.05% SDS, 1 × N-2 supplement [Thermo Fisher]) prior to being added to the assay for testing. A total of 98 µL of RT-QuIC master mix (PBS, 0.12 M NaCl, 1 mM EDTA, 0.06 mM thioflavin T, 20 mM sodium azide, 5 µg/98 µl bovine rPrP) was aliquoted into each well of a 96 well assay plate. Samples to be tested were spiked into designated wells to bring the final assay volume to 100 µL. Assay plates were placed in a Fluostar Optima set to 42 °C during successive shaking (700 rpm) and incubation cycles at 1 min intervals. The fluorescence intensity of each well was measured every 15 min for the duration of the assay. Amyloid seeding was considered to occur when the ThT fluorescence of a well exceeded the threshold value. The threshold value was calculated as 2 times the average fluorescence reading for all negative control replicates on the plate from hour 1 to hour 11.

4.10. Statistical analysis

For RT-QuIC, each tissue/sample was tested in at least 12 replicate wells and the results were analyzed using the method published by Gray (Gray et al., 2016). A receiver operating characteristic (ROC) curve was generated to determine a time cut-off indicating 100% sensitivity and 100% specificity using PRISM v5.04 software (Graphpad). This analysis was conducted using the times at which ThT-signals occurred, as seeded by known BSE+ cases and BSE- controls. ThT-signal times from 3 BSE+ bovines (one of each C-, H-, and L-Type) were pooled for positive control values. Each BSE+ case was tested using 12 replicate wells (n = 36 total BSE+ seeded RT-QuIC reactions). ThT-signal times from 3 BSE- control animals were pooled and used as negative control values (n = 36 BSE- seeded reactions). A cut-off time of ≤36.46 h was calculated for ThT-signals. Differences in ThT signal times between sample types were also assessed by Kruskal-Wallis ANOVA on ranks and differences between mean ThT-signal times seeded by BSE+, BSE-, and Swiss cases were assessed via Cohen's d.

Conflicts of interest

The authors declare no conflict of interests, financial or otherwise.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.brainres.2017.01.015>.

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