Highly sensitive detection of small ruminant BSE within TSE mixes by serial Protein Misfolding Cyclic Amplification

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Running Head: Detection of BSE in TSE mixes

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Abstract

It is assumed that sheep and goats would have consumed the same BSE contaminated meat and bonemeal that was fed to cattle and which precipitated the UK BSE epidemic, which peaked more than twenty years ago. Despite intensive surveillance for cases of BSE within the small ruminant populations of the UK and EU, no instances of BSE have been detected in sheep, and in only two instances has BSE been discovered within goats. If BSE were present within the small ruminant populations it may be at subclinical levels, may manifest as scrapie or may be masked by co-infection with scrapie. To determine whether BSE is potentially circulating at low levels within the European small ruminant populations, highly sensitive assays are required that could specifically detect BSE even within the presence of scrapie prion protein. Here we present a novel assay based on the specific amplification of BSE using the serial Protein Misfolding Cyclic Amplification assay (sPMCA), which specifically amplified low amounts of ovine and caprine BSE which had been mixed into a range of scrapie positive brain homogenates. Detection of BSE prion protein within a large excess of classical, atypical and CH1641 scrapie isolates is demonstrated. In a blind trial this sPMCA based assay specifically amplified BSE within brain mixes with 100% specificity and 97% sensitivity when BSE is diluted into the scrapie brain homogenates at 1% v/v.
Introduction

Transmissible spongiform encephalopathies (TSEs) or prion diseases are a group of fatal neurodegenerative disorders that affect a range of mammalian species including important food production animals and man. The causal agent is proposed to be an abnormal conformer of a host encoded glycoprotein known as PrP\(^C\) (1), which is expressed most highly in tissues of the CNS. This abnormal (disease) conformer, PrP\(^\Sc\), is responsible for the recruitment and conversion of PrP\(^C\) to further molecules of PrP\(^\Sc\). PrP\(^\Sc\) has the propensity to form amyloid fibrils and its formation is thought to be linked to the neurodegeneration of tissues within the CNS. To date PrP\(^\Sc\) is the only validated biomarker for these diseases. Examples of prion diseases are Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, and chronic wasting disease (CWD) in cervids. A further prion disease, Bovine Spongiform Encephalopathy (BSE), emerged in the UK in the mid-1980s as a result of the recycling of TSE infected bovine slaughter offals via meat and bonemeal feed additives within the ruminant feed chain. In the mid 1990’s incontrovertible biological and molecular evidence linked the consumption of BSE contaminated material to a new human prion disease, variant CJD (2,3), of which there have so far been 177 cases within the UK.

Sheep and goats are host to a number of prion strains, categorized into classical, atypical (4) and CH1641-like scrapie (5); each display distinct PrP\(^\Sc\) molecular phenotypes and pathologies. Classical scrapie is likely to be made up of many distinct strains (6). Distinction of small ruminant prion strains is of considerable interest due to the possible presence of BSE and the recognition of a widespread novel prion strain, atypical scrapie, within these species (7). Strain typing methods have been reported that can distinguish classical scrapie from...
experimental ovine BSE and/or CH1641, including bioassay in inbred mice and
immunohistochemical analysis (8). We and others have reported rapid biochemical strain-
typing tests involving analysis of protease-resistant PrPSc. Such PrPSc types differ between
classical scrapie and BSE or CH1641 in terms of their glycoform ratios and the sites of
protease cleavage (9,10). A more difficult diagnostic challenge is the distinction of ovine BSE
from CH1641-like scrapie, as the two strains yield PrPSc indistinguishable by conventional
surveillance assays (11). However, CH1641 and ovine BSE have been distinguished on the
basis of a higher resistance to denaturation shown by ovine BSE (12) or by multiplexed-
antibody detection of protease-resistant PrPSc fragments on western blots (13). We have also
recently demonstrated that ovine BSE can be differentiated from classical, atypical and
CH1641 scrapie isolates by serial Protein Misfolding Cyclic Amplification (sPMCA), a
methodological approach that is thought to replicate, in vitro, the prion conversion event
associated with these diseases and can be used as a sensitive assay for the detection of prions
(14).

BSE prions are readily amplified under a set of defined conditions where all scrapie isolates
tested were refractory to amplification irrespective of PRNP genotype or scrapie strain (15).
Small ruminants are susceptible to experimental oral challenge by the BSE agent (16) and
were very likely fed BSE-contaminated meat and bonemeal before this material was excluded
from ruminant feedstuffs. Despite intense surveillance to date only two instances of BSE
(both in goats) have been detected in small ruminants (17,18) and this very low incidence of
BSE cases in small ruminants remains unexplained. It is possible that BSE-PrPSc may not be
detectable by conventional PrPSc analysis either because it is present at very low levels,
because its molecular phenotype has evolved during interspecies transmission and/or because
it is masked by scrapie during co-infections. Here, we report the development and application of a BSE specific sPMCA assay to an extensive range of BSE/scrapie mixes which could be applied to the detection of BSE within TSE co-infections in small ruminants.

Materials and Methods

TSE Samples:
Scrapie samples (classical ovine and caprine, atypical ovine) from a wide UK geographical distribution, and experimental ovine and caprine BSE brain material were provided by the AHVLA biological archive. CH1641 samples from experimental challenges were provided by Professor Nora Hunter (Roslin Institute). In total 54 individual scrapie isolates were used in combinations with 8 different BSE isolates (see table 1). Samples were prepared as 10% w/v brain homogenates as previously described (15).

sPMCA:
sPMCA was carried out as previously described (15). 10% w/v brain homogenate substrates were prepared from a scrapie-free sheep from a flock with extremely high levels of biosecurity and no history of classical scrapie. Following euthanasia the whole brain was immediately removed and kept on wet ice for up to 3 hours to allow transport back to the laboratory. Brain material was thoroughly cleaned in 1X PBS, the meninges, visible blood vessels and signs of blood contamination were removed. Whole brains were then diced and flash frozen in liquid nitrogen, before storage at -80 °C. To prepare the sPMCA substrates
frozen brain material from animals of either PRNP genotype AHQ/AHQ (alanine, histidine and glutamine at codons 136, 151 and 170 respectively) or VRQ/VRQ PRNP genotype (valine, arginine and glutamine at codons 136, 151 and 170 respectively) were defrosted on ice and then homogenised in a blender at 10% w/v in ice cold buffer consisting of 150 mM NaCl, 4 mM EDTA, pH 8.0, 1% (w/v) Triton X-100 and 1X protease inhibitor solution, (Roche). Blended brain homogenate was then further homogenised by bead-beating for 30 seconds with 1mm glass beads. After 10 minutes centrifugation at 400 x g, clarified 10% brain homogenate substrate was aliquotted and stored at -80 °C until use.

sPMCA reactions were set up by adding the test sample at a 1 in 10 dilution into AHQ/AHQ sPMCA brain homogenate substrate to a final volume of 100 μL. Samples were sealed within 0.2 ml PCR tubes and then placed in an ultrasonicating water bath (model 4000; Misonix) at 37 ºC. Sonications were performed for 40s at 200 W and were repeated once every 30 minutes for 24 hours (one sPMCA round) after which samples were diluted 1 in 3 with fresh substrate brain homogenate of VRQ/VRQ genotype, to a final volume of 100 μl and the sample subjected to a further round of sPMCA. All samples were taken to a total of 5 rounds of sPMCA, using AHQ/AHQ brain substrates at rounds 1, 3 and 5, with VRQ/VRQ substrate used at rounds 2 and 4.

**Analysis of sPMCA products:**

Reaction products were digested with proteinase K (PK) and were then analysed by western blot using the monoclonal antibody SHa31 as described previously (15). Briefly, samples were digested with 50 μg/ml PK in the presence of 0.045% (w/v) SDS for 1 hour at 37 °C. Samples were boiled in 1X NuPAGE SDS-PAGE sample buffer for 5 minutes and then an equivalent to 2 μl of the reaction products were electrophoresed on 12% (w/v) polyacrylamide
gels (pre-cast NuPAGE SDS-PAGE Bis-Tris (Invitrogen)). Samples were transferred to PVDF membranes by electroblotting, then blocked in 3% skimmed milk/PBS. Blots were probed with a SHA31 monoclonal antibody ascites at a dilution of 1/80,000 (Cayman Chemicals) in 0.5% w/v skimmed milk–PBS. After washing, blots were then probed using a secondary goat anti-mouse HRP conjugate at 1/20,000 dilution (DAKO). Blots were visualised using an HRP chemilluminecent substrate (Geneflow), and a Photek imaging system. Confirmatory analysis for single sample replicates were carried out by digestion of samples with PK followed by detection of prion by western blotting using the antibodies P4 at 1/2000 dilution (r-biopharm) and SHA31, on two separate blots. Each blot was additionally loaded with a 2µl sample of 10% brain homogenate from an ovine scrapie and ovine BSE isolate to serve as blotting controls.

Analysis of BSE/Scrapie mixes by western blot

BSE and scrapie samples (single isolates) were made up in proportions from 100% BSE, 0% scrapie in 10% increments to 100% scrapie, 0% BSE. BSE and scrapie isolates were chosen that had approximately equal amounts of PrPSc present as judged by western blot of proteinase K digested brain material. These mixes were analysed on replicate western blots using either the anti prion protein antibodies P4 or SHA31. Blots were evaluated by densitometry of the ratio of each glycan band that made up the protease resistant PrP triplet on the western blots. Values for the monoglycan vs diglycan signal for each sample lane were plotted together with additional BSE and scrapie isolates. The BSE/scrapie mix samples were analysed a total of three times for each mix on three separate western blots.

Densitometry and determination of SHA31/P4 ratio
For densitometry, gel images were measured with Image J software. The lanes on the western blot were defined manually and the lane pixel density was plotted, the areas corresponding to the band peaks were defined so that the background for the lane could be subtracted. The output area under the curve was expressed as an optical density. To remove subjectivity when scoring weak signals after initial SHa31 western blot analysis, a threshold value of 5800 was imposed for samples to be called as positive at the first SHa31 screen. This threshold value was determined empirically by looking at a number of negative control sample lanes blotted and probed in the same way as samples under test. This figure represents the mean plus 3 standard deviations of the SHa31 western blot signal of 32 negative control sPMCA samples. The relative SHa31/P4 ratio is the ratio of the western blot signals produced by amplified sPMCA products when probed with SHa31 and P4 monoclonal antibodies on two separate blots, and is compared to the SHa31/P4 ratio of a control scrapie sample analysed on the same blots (12). This relative ratio is a measure of the presence of the P4 epitope within PrPSc, present at relatively high levels in ovine scrapie, but which occurs at much lower levels in ovine BSE after PK digestion. The absolute ratio of SHa31/P4 was determined for each test sample. This absolute ratio was then divided by the absolute ratio calculated for a scrapie control that was probed on the same blots. This resulted in a relative SHa31/P4 ratio for each sample. A cut off value was derived by repeat analysis of ovine BSE and scrapie samples, which allowed discrimination of BSE from scrapie samples.

**Results**

A standard methodology for the differentiation of BSE from a scrapie is the analysis of the PrPSc signals obtained from dual immunoblotting of samples with antibodies directed to both
the core and towards the N-terminus of the prion protein (9). After PK treatment BSE PrPSc generally lacks the epitope for the more N-terminal antibody P4, whilst maintains reactivity to core antibody. Scrapie PrPSc is reactive to both antibodies. Here dual immunoblotting was able to differentiate samples of 100% BSE from scrapie (Fig 1A), however densitometry of these lanes to generate a SHa31/P4 ratio suggested that this method could only distinguish samples containing up to 80% BSE from scrapie (data not presented). In addition to dual antibody staining, measurement of the glycoform ratios of the monoglycan and diglycan PrPSc species can also be used to discriminate prion strain (19, 20). Densitometry of the western blot signals for the monoglycan and diglycan PrPSc species within brain mixes provided data that was plotted as a scatter plot, this demonstrated that isolates of BSE and scrapie could clearly be differentiated from each other (figure 1B). In this format BSE samples tend to cluster with a greater proportion of diglycosylated PrP than scrapie samples. Also plotted are the mean ratios of BSE/Scrapie mixes (mean of 3 separate analyses). These data demonstrate the insensitivity of current discriminatory assays, and show that at best western blot analysis of brain samples can differentiate BSE/scrapie mixes containing BSE at 60% BSE from natural scrapie isolates.

We have previously detailed the selective in vitro amplification of BSE using specific brain substrates prepared from healthy sheep [15]. Here this methodology was modified for the specific amplification of BSE within BSE/scrapie mixes, a scenario which may mimic brain tissue within mixed prion infections. The sPMCA included a strategy that used ovine brain homogenate substrates from both AHQ/AHQ and VRQ/VRQ PRNP genotype substrates which were used in alternate sPMCA rounds for a total of 5 rounds. This strategy would put greater selection for the specific BSE amplification over the amplification of any scrapie
prions present, as BSE amplifies in both genotypes and scrapie amplification tends to be
genotype restricted. BSE samples (3 isolates) were spiked into different scrapie isolates and
could be detected at dilutions of 1/50, 1/125 and 1/1500 with 100, 90 and 80% sensitivities
respectively at 5 rounds of sPMCA (tables 1S, 2S supplementary information). A diverse set
of scrapie samples from a wide UK geographical distribution were collected, including
classical (ovine and caprine), atypical and CH1641 scrapie isolates. BSE samples came from
experimentally challenged sheep and goats (Table 1). 10% w/v BSE and scrapie brain
homogenates were prepared (10). A sample set consisting of 216 homogenates, 108 being
scrapie only and 108 being scrapie with BSE mixed at 1% v/v, was produced and then
blinded by a third party. Samples (10 μl) were amplified in duplicates by sPMCA for a total
of 5 days. Samples were digested with 50μg/ml Proteinase K and then western blotted using
the monoclonal antibody SHa31. Samples were scored positive if at least 1 reaction gave a
western blot product that was above the blotting mean background +3SDs (Figure 2A).
A single positive replicate from the amplified samples was re-digested and probed with
antibodies SHa31 and P4 on separate western blots. Densitometric scanning of these blots
determined a relative SHa31/P4 ratio. Samples with a relative ratio for SHa31/P4 of 2.9 or
less were defined as scrapie, those samples with ratios above 2.9 were defined as containing
BSE. The figure of 2.9 was derived from the densitometry analysis of 4 scrapie isolates run
on two separate occasions and probed by SHa31 and P4. The mean SHa31/P4 ratio was 1.1,
and the Standard Deviation of these analyses was 0.6. A threshold value was taken as this
mean value plus 3 standard deviations, and this value (2.9) was used as the cut off, below
which a sample was defined as scrapie. Analysis of ovine BSE brain samples on 16 separate
occasions (7 different brains) gave a mean SHa31/P4 ratio of 4.5 (range from 2.9-7.9). On 9
of these analyses, the P4 signal was below the blot threshold and therefore a relative ratio could not be determined for these samples on these occasions. Where samples were positive by SHa31 western blot, but did not produce a measurable P4 reactive signal, samples were regarded as being BSE positive.

Across all amplifications, 32 negative control sPMCA samples were tested (substrate only) and all were negative, and 31 out of 32 positive controls (amplifying 0.1µl ovine BSE brain only) were positive. Blinded sample results are summarised in Figures 2 and 3: 108 of 108 scrapie-only samples were negative for BSE, whilst 105 of 108 of BSE spiked scrapie samples produced BSE-positive sPMCA product. BSE from both goat and sheep origin was amplified under these conditions. Of the 3 BSE-spiked samples that did not amplify BSE, two failed to give an amplification product and one amplified but had a SHa31/P4 ratio indicative of scrapie.

Discussion

It is possible that within TSE co-infections the amount of BSE that may be present could be in low amounts and may vary in levels between tissue types, which would require very sensitive assays for detection. Strain typing tests based on western blot methodology are largely only applicable to pure strains [9]. We demonstrate here that these methods were at best able to differentiate a BSE/scrapie mix in a 60:40 proportion from isolates of scrapie. For the detection of BSE that may be present at lower levels than this, which may be present within co-infection requires assays that have far higher levels of sensitivity. The sPMCA methodology described here has very high levels of sensitivity and specificity for BSE that can be used to differentiate BSE prion protein from classical, CH1641 and atypical scrapie.
prion protein even when these different scrapie brain materials were present at a 100-fold excess compared to BSE. This study was carried out using a comprehensive collection of scrapie isolates within a range of PRNP genotypes. In a blind trial of 216 such samples the BSE sPMCA test showed a specificity of 100% (all 108 negatives samples tested negative) and a sensitivity of 97.2% (105 of 108 positive samples tested positive). The positive predictive value for the assay was 100% (all 105 of samples that tested positives were true positives) and the negative predictive value was 97.3% (108 of the 111 samples that tested negative were true negatives). The assay should prove extremely useful alongside current screening methodology in the surveillance of small ruminants for BSE including the analysis of scrapie positive samples for co-infection with BSE. This test could be applied to new or historical cases of small ruminant TSE presenting with confusing pathology or molecular phenotypes (17). The methodology could also be used to inform risk assessments to human health of animal products from experimental mixed TSE infections.

Acknowledgments

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References


2010 Lesion profiling at primary isolation in RIII mice is insufficient in distinguishing BSE from classical scrapie. Brain Pathol. 20: 313-22.
Table 1. Scrapie isolates tested in mixes with BSE.

<table>
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<tr>
<th>Scapie type¹</th>
<th>Genotype²</th>
<th>Number of isolates³</th>
<th>Number of BSE isolate spikes⁴</th>
<th>Number of analyses⁵</th>
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<td>VRQ/VRQ</td>
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<tr>
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<td>6</td>
<td>12</td>
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<td>6</td>
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<td>Totals</td>
<td></td>
<td>54</td>
<td>108</td>
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¹Scrapie isolates that were used in this study are listed as either classical, atypical, CH1641 (all ovine) or caprine scrapie.

²PRNP genotype of the scrapie isolate at the codon positions 136, 154, 171.

³The number of scrapie isolates of each individual genotype

⁴The number of independent isolates of either ovine or caprine BSE that were spiked into scrapie samples of that particular genotype. Each scrapie isolate was spiked with two distinct BSE samples. In total 6 ovine BSE isolates were used (4 of ARQ/ARQ, 2 of AHQ/AHQ genotype) and two caprine BSE isolates were used (ARQ/ARQ).
The number of individual sample mixes for each genotype that were prepared and analysed. Total number of BSE/scrapie mixes analysed was 108, in addition 108 samples consisting of just the scrapie samples alone were also tested.

**Figure 1.** Samples of BSE and Scrapie brains were PK digested, mixed in different proportion (100 to 0% BSE in 10% increments as indicated) and then western blotted with anti-prion antibodies SHa31 or P4. BSE immunolabelled with SHa31 but not P4 (each mixture ratio was prepared and analysed 3 times on 3 separate western blots, a representative example of these blots is shown in Panel A; MM- molecular weight markers, (20 and 30kDa).

The relative abundance of both the monoglycan and diglycan species within each of these mixed samples (SHa31 blot) was calculated by densitometry, these values were plotted as a scatter plot using the mean value of three analyses (Panel B). BSE/scrapie mixes are plotted (black diamonds), labelled as the percentage of BSE content (v/v) with error bars depicting the +/- standard error of these means. Individual experimental isolates of ovine BSE (grey squares) and isolates of classical ovine scrapie (grey triangles) were plotted as a function of their monoglycan and diglycan signals on a SHa 31 western blot. Scrapie and BSE samples clearly cluster on different sides of the plot indicating the higher abundance of diglycosylated PrP in PK treated BSE samples than in scrapie.
**Figure 2.** sPMCA amplification of BSE brain homogenate spiked into a 100-fold excess of scrapie. A: representative western blot from the blind trial depicting duplicate sPMCA analyses of four separate BSE/scrapie sample mixes labelled 1-4. 1: ovine BSE in ovine scrapie, 2: ovine BSE in ovine atypical scrapie, 3: classical ovine scrapie only, 4: atypical ovine scrapie only; NEG are 4 negative control sPMCA amplifications that contained no TSE; +BSE are positive control amplifications that were spiked with 0.1µl of 10% ovine BSE brain only. Blots were developed with SHa31 antibody. B: representative examples of putative BSE sPMCA-positive samples from the blind trial (labeled 1-9) probed with both SHa31 (upper panel) and P4 (lower panel). Lanes 1,3,5,6 are ovine BSE spiked into ovine classical scrapie. Lanes 2&4 are ovine BSE in CH1641 scrapie, lanes 7&8 are ovine BSE in atypical scrapie, lane 9 is caprine BSE in caprine scrapie. Blots A and B: +S, PK-digested scrapie brain blotting control, +B, PK-digested BSE brain blotting control, M, molecular mass markers: 20, 30kDa.

**Figure 3.** Summary of the relative SHa31/P4 western blot ratios for sPMCA amplified products. Upper panel, the 108 scrapie only samples that were subject to sPMCA. A single sample from a ARH/VRQ sheep amplified but its relative SHa31/P4 ratio was indicative of scrapie (below the 2.9 cut off shown as grey horizontal line). All other 107 samples did not produce measurable levels of sPMCA product as assessed by western blotting, and a relative SHa31/P4 ratio is not recorded. Lower panel, 108 BSE/scrapie mixes where BSE brain was present at 1% that of the scrapie brain homogenate were analysed. Two samples did not produce sPMCA product and are depicted on the chart with no relative SHa31/P4 ratio. A single sample produced an sPMCA product but was reactive against both SHa31 and P4.
giving a relative SHa31/P4 ratio that was indicative of scrapie (below the SHa31/P4 2.9 relative ratio cut off). SHa31/P4 signals for samples amplifying BSE have been cropped to a maximum signal of 3.4, for illustrative purposes.