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RESEARCH PAPER

In vitro amplification of H-type atypical bovine spongiform encephalopathy by protein misfolding cyclic amplification

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ABSTRACT. The *in vitro* amplification of prions by serial protein misfolding cyclic amplification has been shown to detect PrP^{Sc} to levels at least as sensitive as rodent bioassay but in a fraction of the time. Bovine spongiform encephalopathy is a zoonotic prion disease in cattle and has been shown to occur in 3 distinct forms, classical BSE (C-BSE) and 2 atypical BSE forms (L-BSE and H-BSE). Atypical forms are usually detected in asymptomatic, older cattle and are suggested to be spontaneous forms of the disease. Here, we show the development of a serial protein misfolding cyclic amplification method for the detection of H-BSE. The assay could detect PrP^{Sc} from 3 distinct experimental isolates of H-BSE, could detect PrP^{Sc} in as little as 1×10^{-12} g of brain material and was highly specific. Additionally, the product of serial protein misfolding cyclic amplification at all dilutions of seed analyzed could be readily distinguished from L-BSE, which did not amplify, and C-BSE, which had PrP^{Sc} with distinct protease K-resistance and protease K-resistant PrP^{Sc} molecular weights.

KEYWORDS. atypical prions, bovine spongiform encephalopathy, H-type BSE, PMCA

INTRODUCTION

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are progressive, fatal diseases that affect a range of mammalian species, including man. It is now widely accepted that the central event in these diseases is the misfolding of the benign cellular prion protein

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(PrP^C) into a disease-associated isoform (PrP^{Sc}).[1] Over a very protracted presymptomatic phase, PrP^{Sc} converts host PrP^C into the misfolded form and accumulates, particularly in the central nervous system (CNS) tissues. This disease process leads to neurodegeneration, clinical symptoms and death.

It is usual for TSEs to be host restricted with the notable exception of bovine spongiform encephalopathy (BSE). BSE was first described in cattle in the mid 1980s and led to the BSE epidemic in cattle in the UK. The BSE agent has also been identified as causing disease in humans (variant Creutzfeld Jakob disease, vCJD), exotic ungulates, wild and domestic cats and goats. It is likely that the BSE agent was transmitted to these different host species via contaminated feed/food. This unusually wide range of host species for BSE would indicate that this form of prion is relatively promiscuous and can replicate within hosts having a diverse range of PrP^C primary sequences and concomitant tertiary structures.

The molecular properties of the PrP^{Sc} generated during classical BSE infections (so-called C-BSE) produces a protease-resistant core with a very characteristic molecular weight. C-BSE also produces consistent *in vivo* pathology in bovines[2] as well as upon passage in rodent models even with BSE from various host species.[3] Two further prion diseases have been identified in cattle due to their divergence from the consistent molecular and pathological traits of C-BSE, namely L-type and H-type BSE. L-BSE (or bovine amyloidotic spongiform encephalopathy, BASE) was first identified by distinctive amyloid plaques and PrP^{Sc} distribution within the brain compare with C-BSE.[4] L-BSE also produces a PK-resistant PrP^{Sc} with a lower molecular weight than C-BSE together with a lower proportion of the diglycosylated form.[4] H-BSE produces a PK-resistant PrP^{Sc} with a high molecular weight compare with C-BSE,[5,6,7] Both forms of the disease were identified by active surveillance in asymptomatic cattle and these animals were usually 8 y or older leading to speculation that these may be spontaneous diseases.[8]

Both atypical forms of BSE have been transmitted to mice expressing the human PrP^C gene as well as non-human primates at rates exceeding C-BSE, highlighting the possibility that these could be zoonotic agents.[9,10,11]

Taken together, atypical BSE diseases are potentially zoonotic agents that are usually present in asymptomatic cattle. Therefore there is a need for highly sensitive assays to detect these forms of BSE, including during pre/subclinical phases where prion may be present at relatively low levels. Several reports have now demonstrated that *in vitro* replication assays may provide such tests. The protein misfolding cyclic amplification (PMCA) method is based on iterative rounds of sonication and incubation to replicate very low levels of PrP^{Sc} seed within PrP^C substrate (usually a brain homogenate).[12] The sonication step is thought to break prion aggregates into smaller units that can efficiently seed the conversion of PrP^C into PrP^{Sc} during the incubation period. Prion is amplified over multiple (24-48) cycles of sonication/incubation (a round) and when serial PMCA (sPMCA) is performed, for each subsequent round of amplification the substrate is replenished by diluting the PMCA products into fresh substrate.[13] The resulting amplified PK-resistant PrP^{Sc} is then usually detected in western blots. Quaking induced conversion (QuIC) is an alternative *in vitro* assay.[14] This is based on the ability of PrP^{Sc} to seed the conversion of a recombinant PrP substrate into amyloid fibrils. The method relies on iterative rounds of incubation and vigorous shaking, fibrils form during the incubation steps and are fragmented into smaller seeds by shaking. The QuIC amyloid products are usually monitored by thioflavin T fluorescence in real time, although proteinase K (PK) resistant products can also be analyzed by western blotting.[15]

To date, PMCA has been used for the high sensitivity detection of L-BSE derived from experimentally infected macaques although the products were not distinguished from other types of BSE.[16] QuIC has also been used to amplify both L-BSE and H-BSE from infected cattle. However, it was not possible to distinguish the BSE types when present at relatively low concentrations.[15]

Here, we have used sPMCA to demonstrate the very high sensitivity detection of H-BSE that can readily distinguish H-BSE from L-BSE or C-BSE regardless of the PrP^{Sc} seed concentration.

RESULTS

The amplification of atypical H-BSE prions by sPMCA and subsequent analysis of the products was performed by varying up to 4 conditions: the source of substrate (including PrP^{C} primary sequence), the sonication duration, the presence or absence of Teflon beads and the concentration of PK used to digest sPMCA products (Table 1). The first optimisation step looked at a single sonication time (40s) in the absence of Teflon beads using 4 substrates derived from bovine, ovine AHQ/AHQ (corresponding to codons 136, 151, and 170 in the *PRNP* gene respectively), ovine ARQ/ARQ and ovine VRQ/VRQ brains. Out of 2 replicate analyses for each substrate, only a single reaction with the bovine substrate gave detectable PrP^{Sc} triplet (Fig. 1A). Repeat analysis of these conditions with bovine substrate demonstrated that all 5 replicates gave low but detectable levels of PrP^{Sc} product (Fig. 1B). When sonication times of 30, and 20 s were used with bovine substrate, 30 s gave low levels of PrP^{Sc} in all 5 replicates and 20 s gave low levels of PrP^{Sc} in 2 out of 5 replicates. In the presence of Teflon beads with bovine substrate, all 3 sonication times failed to produce any PrP^{Sc}. The products of amplification of H-BSE in bovine substrate with all sonication times were digested with 5, 10 or 50 μg/ml. While weak PrP^{Sc} signals were detected on western blots with the highest protease concentration, all of these positive amplifications yielded higher levels of defined PrP^{Sc} triplet at the lower 2 concentrations over background undigested PrP (Fig. 1B and data not shown). It was noted that at these lower protease concentrations, a single band for substrate PrP^{C} that was between the molecular weights for di- and mono-glycosylated PK-resistant PrP^{Sc} could be observed, as

TABLE 1. Optimisation of the replication of H-BSE in sPMCA.

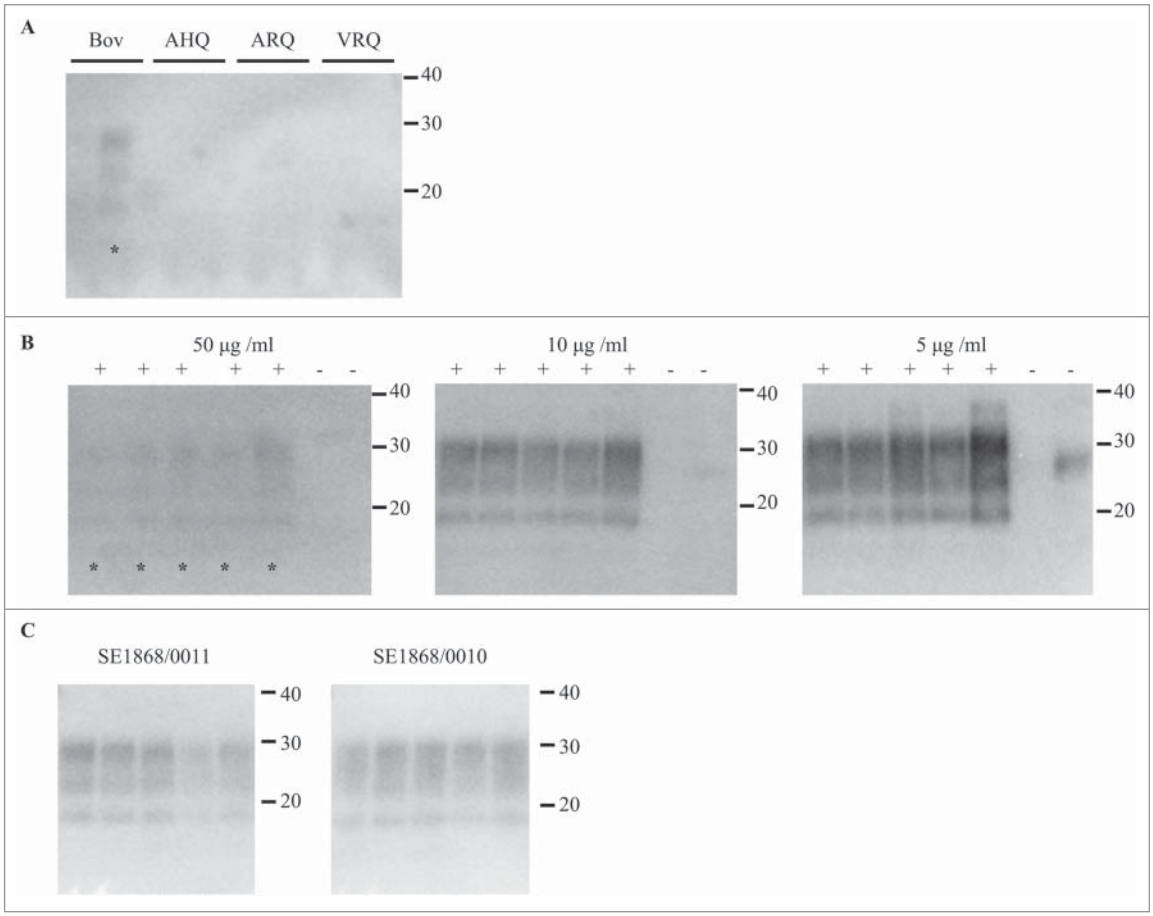
Sonication time (s)	Presence of Teflon beads	Substrate	[PK] (μg/ml)	Amplification effciency[1]
Phase 1: optimisation of substrate				
40	–	bovine	50	1/2[2]
40	–	ovine AHQ	50	0/2
40	–	ovine ARQ	50	0/2
40	–	ovine VRQ	50	0/2
Phase 2: optimisation of sonication time				
40	–	bovine	50	5/5[2]
30	–	bovine	50	5/5[2]
20	–	bovine	50	2/5[2]
Phase 3: presence of Teflon beads				
40	+	bovine	50	0/5
30	+	bovine	50	0/5
20	+	bovine	50	0/5
Phase 4: digestion of products with different protease concentrations				
40	–	bovine	10	5/5
			5	5/5
30	–	bovine	10	5/5
			5	5/5
Phase 5: specificity of the optimised conditions				
40	–	bovine	10	0/20[3]

[1]Number of amplification reactions producing protease-resistant PrP^{Sc}/total number of reactions

[2]These were relatively weak signals on western blots

[3]Control sPMCA reactions not containing any PrP^{Sc} seed

FIGURE 1. Amplification of H-BSE by sPMCA. A single isolate of H-BSE (SE2053/0089) was amplified over 5 d by sPMCA in duplicate in ovine (*PRNP* genotypes AHQ, ARQ or VRQ) or bovine (bov) substrate and products digested with 50 μg/ml PK (A; * weak signal detected for PrP^{Sc} triplet). The same H-BSE isolate was amplified in 5 replicate reactions using bovine substrate and products after 5 d of amplification were digested with 50, 10 or 5 μg/ml PK (B; * weak signal detected for PrP^{Sc} triplet,+ reaction containing H-BSE seed, - reaction not containing any H-BSE seed). Two further isolates of H-BSE were amplified in 5 replicates in bovine substrate and products digested with 10 μg/ml PK (C). All sPMCA reactions used 40 s sonication durations and digested products were analyzed on western blots using anti-PrP antibody SHa31. Molecular weight markers are indicated.



previously reported.[17] The optimal conditions: bovine substrate, 40 s sonication times without Teflon beads and 10 μg/ml PK digestion of sPMCA products were used in all subsequent experiments. Three experimental isolates of H-BSE were available for analysis and all 3 amplified under these sPMCA conditions when analyzing 10 μl of a 10^{-2} dilution of brain homogenate (equivalent to 100 μg of brain material) with 5 out of 5 replicates amplified for each (Fig. 1B and C). Similar amplification of C-BSE consistently yielded PrP^{Sc} but 3 isolates of L-BSE were not amplified under these conditions (data not shown). Dilution series of H-BSE and C-BSE were analyzed (Table 2, Fig. 2). For C-BSE, 2 samples were analyzed and the limit of detection of PrP^{Sc} was when analyzing a 10^{-6} or 10^{-7} dilution of brain (equivalent to 10 or 1 ng of brain material). For H-BSE the limit of detection was at least as

TABLE 2. Limit of detection of H-BSE amplification by sPMCA.

Brain tissue dilution[a]	H-BSE SE2053/0089	H-BSE SE1868/0011	C-BSE pool 1[b]	C-BSE SE1762/0013
10^{-2}	ND	2/2	2/2	2/2
10^{-3}	2/2	2/2	2/2	2/2
10^{-4}	2/2	2/2	1/2	2/2
10^{-5}	2/2	2/2	2/2	1/2
10^{-6}	2/2	2/2	1/2	1/2
10^{-7}	2/2	2/2	1/2	0/2
10^{-8}	2/2	0/2	0/2	0/2
10^{-9}	2/2	0/2	0/2	0/2
10^{-10}	1/2	ND	ND	ND
10^{-11}	0/2	ND	ND	ND
10^{-12}	0/2	ND	ND	ND

[a]10^{-2} dilution contains 100 μg of brain tissue and is the equivalent of using 1 μl of a 10% (w/v) brain homogenate as the seed in a 100 μl sPMCA reaction.

[b]The sample was a pool of 10 bovine BSE isolates mixed in equal volumes

sensitive, PrP^{Sc} could be detected in the brain of 2 experimental isolates at 10^{-7} or 10^{-10} dilutions (equivalent to 1 ng or 1 pg of brain material). The analysis of 20 replicates that contained no PrP^{Sc} seed did not produce any detectable PrP^{Sc} triplet, only a single undigested PrP^{C} band as described above (data not shown). It was clearly evident that amplification products from H-BSE seed were distinguishable from those from C-BSE seed across the range of seed concentrations used. The H-BSE and C-BSE products of sPMCA could be distinguished due to their PK-resistance by determining the ratio of PrP^{Sc} signal produced when digesting samples with 10 or 50 μg/ml PK ($p = 0.008$ using a 2-tailed, unpaired Student's t test; Fig. 3). The H-BSE and C-BSE products of amplification also

FIGURE 2. Estimation of the limit of detection of H-BSE by sPMCA. H-BSE isolates SE2053/0089 (A) and SE1868/0011 (B) were amplified by sPMCA using bovine substrate and 40 s sonication times over 5 d. The dilution of brain material used to seed sPMCA reactions is indicated. Products of amplification were digested with 10 μg/ml PK before western blot analysis and probing with anti-PrP antibody SHa31. Molecular weight markers are indicated.

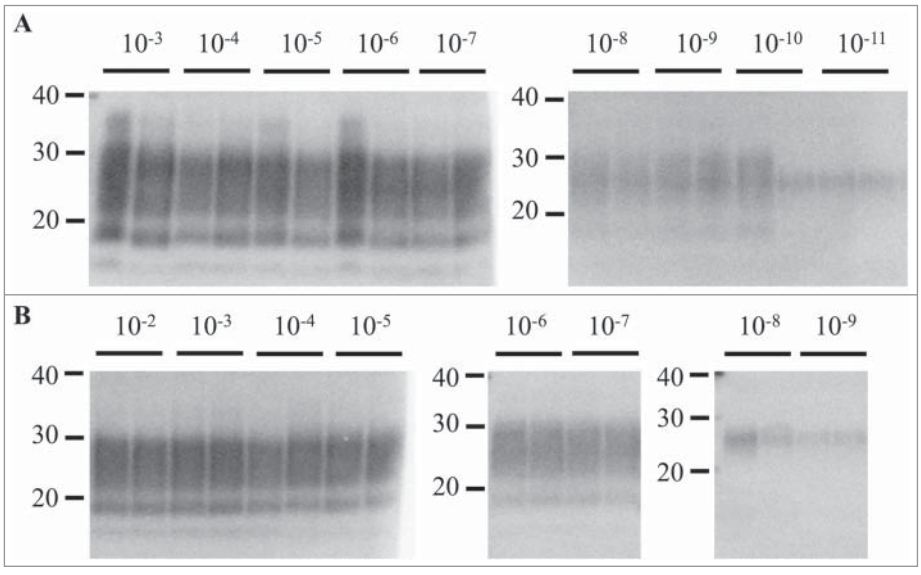
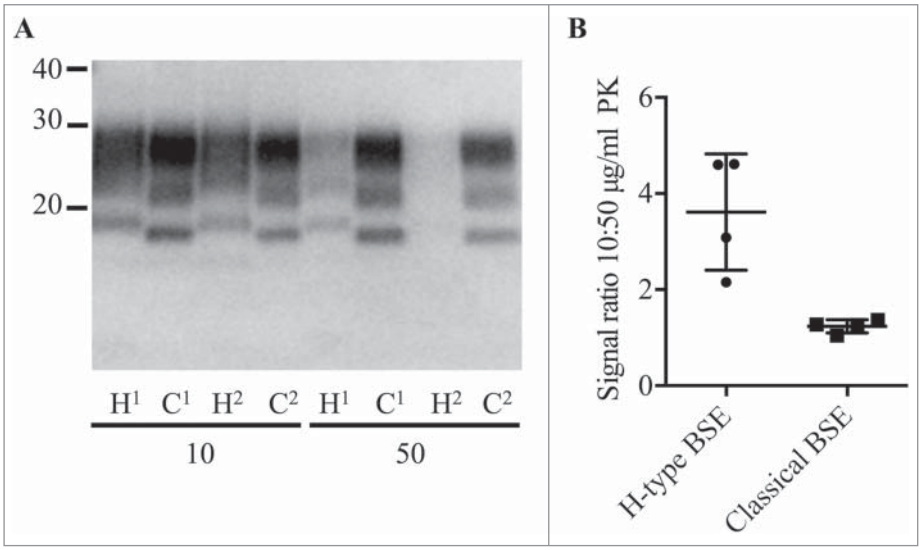


FIGURE 3. Differentiation of amplified H-BSE and C-BSE by distinct protease resistance. H-BSE (SE2053/0089) and C-BSE (pool-1) samples were each amplified by sPMCA using bovine substrate and 40 s sonication times over 5 d. Products of amplification were digested with 50 or 10 μg/ml PK (as indicated) before western blot analysis and probing with anti-PrP antibody SHa31 (A). The spikes used for sPMCA of H-BSE were dilutions at 10^{-2} (100 μg of brain material, H^1) or 10^{-7} (1 ng of brain material, H^2) of brain material. The spikes used for sPMCA of C-BSE were dilutions at 10^{-2} (100 μg of brain material, C^1) or 10^{-5} (100 ng of brain material, H^2) of brain material. The ratio of PrP^{Sc} western blot signals for sPMCA products at the 2 PK concentrations are shown when analyzing 2 H-BSE isolates (SE2053/0089 and SE1868/0011) and 2 C-BSE samples (pool-1 and SE1762/0013) at high and low spike concentrations (B). Molecular weight markers are indicated.

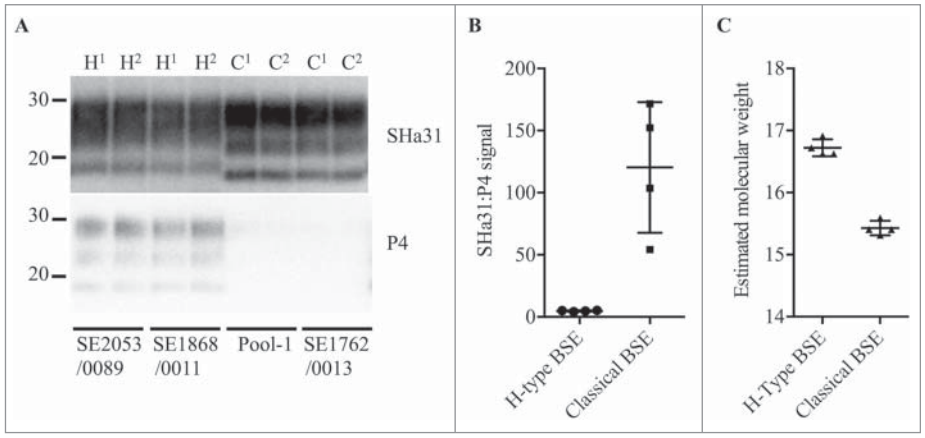


differed in the molecular weight of the unglycosylated PrP^{Sc} band ($p<0.0001$ using a 2 tailed, unpaired Student's t test; Fig. 4C) which was readily identified by the level of binding to the antibody P4 (ratio values analyzed in a 2 tailed, unpaired Student's t test $p = 0.0046$; Fig. 4A and B).

DISCUSSION

The detection of PrP^{Sc} is a surrogate marker for prion diseases and has formed the basis of veterinary screening tests for ruminants. C-BSE in cattle is the only zoonotic prion disease recorded to date and as such the testing of food production animals is driven by the requirement to detect the C-BSE agent and minimize its risk to human health. Screening tests applied to cattle use post mortem brain tissue samples from which to detect PrP^{Sc} and rely on the presence of relatively high levels of PrP^{Sc} in the caudal region of the brainstem. Such tests may not be sensitive enough to detect PrP^{Sc} present at low levels during relatively early stages of disease incubation. When considering the atypical L-BSE and H-BSE diseases of cattle, they have been assessed in both non-human primate and transgenic mouse bioassays (with mice transgenic for human *PRNP*) and both model systems indicate that H-BSE and L-BSE may have increased zoonotic potential compare with C-BSE.[9,10,11] The detection of all types of BSE is therefore of significant importance. Both L-BSE and H-BSE have been identified in mainly asymptomatic animals of a relatively advanced age, ~12 y on average[18] compare with the estimated incubation period for cattle naturally infected with C-BSE, which is 5.0–5.5 y.[19,20] It has been suggested that atypical BSE types may be spontaneous diseases, therefore explaining the detection of PrP^{Sc} in mainly

FIGURE 4. Differentiation of amplified H-BSE and C-BSE by distinct PK cleavage sites. Two samples each of H-BSE and C-BSE samples were amplified by sPMCA using bovine substrate and 40 s sonication times over 5 d. Products of amplification were digested with 10 μg/ml PK before western blot analysis and probing with anti-PrP antibody SHa31 or P4 (A). The spikes used for sPMCA of H-BSE were dilutions at 10^{-2} (100 μg of brain material, H^1) or 10^{-7} (1 ng of brain material, H^2) of brain material. The spikes used for sPMCA of C-BSE were dilutions at 10^{-2} (100 μg of brain material, C^1) or 10^{-5} (100 ng of brain material, H^2) of brain material. The ratio of PrP^{Sc} western blot signals for sPMCA products detected by the 2 antibodies are shown (B). The molecular weight of the unglycosylated PrP^{Sc} band for each sample is also shown (C). Molecular weight markers are indicated.



older animals.[8] The presence of relatively low levels of PrP^{Sc} in atypical BSE cases, compared with C-BSE, is likely for most age groups of cattle. Therefore the detection of PrP^{Sc} by conventional biochemical tests used in routine surveillance would be more challenging in atypical BSE cases. Given the high zoonotic potential of atypical BSE types it is important to develop very high sensitivity tests to detect the concomitant PrP^{Sc} in routinely sampled tissue. Such tests would not only be applicable to routine surveillance testing but also to determine *in vivo* dissemination of atypical prions to inform risk assessments of bovine material entering the human food chain. To this end, several studies have looked to develop *in vitro* prion amplification assays to detect very low levels of atypical BSE prions. Murayama and coworkers used wild type mouse brain as sPMCA substrate supplemented with a cocktail of polyanions (polyadenylic acid salt (Poly-A), heparin and sodium polyphosphate) and L-arginine ethylester.[16] The method included sPMCA amplifications over 280 hours and could detect L-BSE down to a 10^{-10} dilution of brain homogenate. The method could detect L-BSE in cerebrospinal fluid, saliva, urine and plasma from macaques experimentally infected with L BSE. Prion was detected in saliva and urine in preclinical samples from 1 of 2 infected animals; and in CSF samples taken preclinical in both animals.

Several studies have looked to use QuIC to amplify both L-BSE and H-BSE and have utilised recombinant hamster, sheep, human and bank vole PrP as substrates.[15,21,22]

Human and hamster PrP consisting of residues 23–231 and hamster PrP residues 90–231 were found to only amplify L-BSE but not C-BSE and could detect L-BSE down to 10^{-6} to 10^{-7} dilutions of brain homogenate, which they report is at least as sensitive as mouse bioassay[21,22] and approximately 4 orders of magnitude more sensitive than commercial immunoassays.[15] Using bank vole recombinant PrP as substrate allowed the amplification of H-, L- and C-BSE. H-BSE could be detected down to 10^{-8} to 10^{-9} dilution of brain homogenate and H-BSE and L-BSE could be detected in the CSF from clinically affected cows.[15]

Masujin and coworkers propose a scheme to use QuIC to distinguish H- L- and C-BSE infections.[15] The proposed method analyses samples simultaneously in bank vole and ovine ARR substrate on the same QuIC plate. C-BSE is only amplified in bank vole substrate and, for L-BSE and H-BSE, the comparative lag phases of amplification are compared for each substrate, with L-BSE having a relatively shorter lag phase compare with H-BSE in ARR substrate, and the reverse being the case in bank vole substrate. The test samples are run alongside positive control samples of each BSE type for comparison. The method was shown to be able to distinguish BSE types for dilutions between 10^{-3} and 10^{-5} of brain homogenate from clinically affected cows but could not differentiate BSE types at lower concentrations.

In summary, *in vitro* amplification methods have been established for L-BSE and H-BSE prions with higher sensitivity than transgenic mouse bioassay ($\sim 10^{-5}$ dilution of brain homogenate). However, at present sPMCA can be used to only detect L-BSE and while the QuIC method can detect both L- and H-BSE, at lower levels of prion ($< 10^{-5}$ dilution of brain homogenate) the BSE types cannot be distinguished.

Here, we developed a sPMCA method to detect H-BSE. The most optimal conditions used 40s sonication durations and bovine PrP^{C} substrate. The assay amplified all isolates of H-BSE and C-BSE tested and could detect H-BSE down to 10^{-7} and 10^{-10} dilution of brain homogenate. L-BSE was not amplified under the conditions used and the H-BSE and C-BSE products of amplification could be readily distinguished by the molecular weight of their unglycosylated PrP product or by the relative PK-resistance of the PrP^{Sc}. The molecular weight differences between PrP^{Sc} from H-BSE and C-BSE is a trait seen in both the brain derived PrP^{Sc} and also the sPMCA products. It has also been previously reported that H-BSE has a lower PK resistance than C-BSE[5] and this trait seems to be exaggerated in the sPMCA products. Importantly, the ability to identify H-BSE in sPMCA products was maintained across all of the dilution ranges of seed analyzed (down to 10^{-7} dilution of brain homogenate) indicating that the assay can detect and strain type very low levels of H-type BSE.

The present study describes a sPMCA method for the amplification of H-BSE PrP^{Sc} that has very high sensitivity and can distinguish H-BSE from other BSE types. Given these properties, the assay has the potential to describe the *in vivo* dissemination of H-BSE PrP^{Sc} in infected animals across the time-course of infection, in order to define tissues that harbour H-BSE prions.

METHODS

Samples

All brain samples from BSE-affected animals were taken from the hindbrain and were provided from the Animal and Plant Health Agency (APHA) biological archive. H-BSE and L-BSE samples were from experimentally infected animals, C-BSE samples were from natural infections. Three samples each of H-BSE, L-BSE and C-BSE were available for use in the study: H-BSE isolates were SE2053/0089, SE1868/0010 and SE1868/0011; L-BSE isolates were SE1868/0008, SE1868/0009 and SE2053/0106; C-BSE samples were SE1762/0013, SE2053/0055 and BSE pool 1. The latter was a pool of 10 bovine BSE isolates mixed in equal volumes: PG00091/04, PG00034/04, PG01184/02, PG01257/02, PG01412/02, PG00345/03, PG00365/03, PG00449/03, PG00487/03 and PG00572/03. Samples were prepared as 10% (w/v) brain homogenates as previously described.[23] Brain samples from TSE-free animals were provided by the APHA.

sPMCA

Substrate was prepared from a single Holstein Friesian cow, or sheep with *PRNP* genotypes AHQ/AHQ (corresponding to codons 136, 151, and 170, respectively), VRQ/VRQ or ARQ/ARQ as previously described.[24] sPMCA was performed basically as previously described[23] but with some modifications. Each sPMCA reaction was set up by adding the test

sample (10 μl of a dilution of brain homogenate) into substrate to a final volume of 100 μl. Samples were sealed within 0.2 ml PCR tubes and then placed into an ultrasonicating water bath (model 4000; Misonix) at 37°C. Sonications were performed for 20, 30 or 40 s at 200 W and repeated once every 30 min for 24 h (one sPMCA round), after which, the samples were diluted 1 in 3 with fresh substrate brain homogenate to a final volume of 100 μl, and the samples were subjected to a total of 5 rounds of sPMCA. sPMCA was also performed in the presence or absence of 3 Teflon beads per tube.[25]

Western Blots

Samples were digested with PK and then analyzed by western blotting using the monoclonal antibodies SHa31 or P4 as described previously.[23] Briefly, samples were digested with 5, 10 or 50 μg/ml PK in the presence of 0.045% (w/v) SDS for 1 h at 37°C. Samples were boiled in 1x NuPAGE SDS-PAGE sample buffer for 5 min, and then an equivalent to 5 μl of the sPMCA reaction products was electrophoresed on 12% (w/v) polyacrylamide gels (precast NuPAGE SDS-PAGE Bis-Tris; Invitrogen). Samples were transferred to polyvinylidene difluoride membranes by electroblotting and then blocked in 3% (w/v) skimmed milk-PBS. Blots were probed with SHa31 or P4 monoclonal antibody at a dilution of 1/80,000 for SHa31(Cayman Chemicals) or 1/1,000 for P4 (R-Biopharm) in 0.5% (w/v) skimmed milk-PBS. After washing, the blots were probed using a secondary goat anti-mouse horseradish peroxidase (HRP) conjugate at a 1/20,000 dilution (Dako). Blots were visualized using an HRP chemiluminescent substrate (Geneflow) and a Photek imaging system.

For densitometry, gel images were measured with ImageJ software and the lane pixel densities were plotted, the signal maxima for each band was used (molecular weight analysis) or the total areas corresponding to the band peaks were defined and the background for each lane was subtracted (all other analysis). When calculating molecular weights, the migration distance of proteins through the SDS-PAGE gel was measured and a standard curve generated using the migration distances of the 60, 50, 40, 30 and 20 kDa molecular mass standards (NuPAGE Magicmark, Invitrogen). Using linear regression analysis, the molecular weight of unglycosylated PrP^{Sc} was then calculated from the curves using Graphpad prism software. The SHa31/P4 ratio for a sample was calculated as the ratio of the western blot signals produced by samples when probed with SHa31 and P4 monoclonal antibodies on 2 separate blots. This is a measure of the presence of the P4 epitope within PrP^{Sc} after PK digestion, the epitope is present at relatively high levels in ovine scrapie isolates and H-BSE, but at much lower levels in C-BSE and L-BSE isolates.[26] The ratio of PK-resistant PrP^{Sc} when samples were digested at 10 and 50 μg/ml PK were determined using densitometry to determine total PrP^{Sc} signals as described above.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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