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 PrPSc 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 PrPSc from 3 distinct experimental isolates of H-BSE, could detect PrPSc 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 PrPSc with distinct protease K-resistance and protease K-resistant PrPSc 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...
(PrPC) into a disease-associated isoform (PrPSc). Over a very protracted presymptomatic phase, PrPSc converts host PrPC 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 PrPC primary sequences and concomitant tertiary structures.

The molecular properties of the PrPSc 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 as well as upon passage in rodent models even with BSE from various host species. 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 PrPSc distribution within the brain compare with C-BSE. L-BSE also produces a PK-resistant PrPSc with a lower molecular weight than C-BSE together with a lower proportion of the diglycosylated form. H-BSE produces a PK-resistant PrPSc with a high molecular weight compare with C-BSE. 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.

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

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 PrPSc seed within PrPC substrate (usually a brain homogenate). The sonication step is thought to break prion aggregates into smaller units that can efficiently seed the conversion of PrPC into PrPSc 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. The resulting amplified PK-resistant PrPSc is then usually detected in western blots. Quaking induced conversion (QuIC) is an alternative in vitro assay. This is based on the ability of PrPSc 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.

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. 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.

AMPLIFICATION OF H-BSE BY PMCA
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 PrPSc 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 PrPSc 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 PrPSc triplet (Fig. 1A). Repeat analysis of these conditions with bovine substrate demonstrated that all 5 replicates gave low but detectable levels of PrPSc product (Fig. 1B). When sonication times of 30, and 20 s were used with bovine substrate, 30 s gave low levels of PrPSc in all 5 replicates and 20 s gave low levels of PrPSc in 2 out of 5 replicates. In the presence of Teflon beads with bovine substrate, all 3 sonication times failed to produce any PrPSc. 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 PrPSc signals were detected on western blots with the highest protease concentration, all of these positive amplifications yielded higher levels of defined PrPSc 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 PrPSc that was between the molecular weights for di- and mono-glycosylated PK-resistant PrPSc could be observed, as

| Sonication time (s) | Presence of Teflon beads | Substrate | [PK] (μg/ml) | Amplification efficiency¹ |
|---------------------|--------------------------|-----------|--------------|--------------------------|
| Phase 1: optimisation of substrate | – | bovine | 50 | 1/2² |
| 40 | – | ovine AHQ | 50 | 0/2 |
| 40 | – | ovine ARQ | 50 | 0/2 |
| 40 | – | ovine VRQ | 50 | 0/2 |
| Phase 2: optimisation of sonication time | – | bovine | 50 | 5/5² |
| 40 | – | bovine | 50 | 5/5² |
| 30 | – | bovine | 50 | 5/5² |
| 20 | – | bovine | 50 | 5/5² |
| Phase 3: presence of Teflon beads | + | bovine | 50 | 0/5 |
| 40 | + | bovine | 50 | 0/5 |
| 30 | + | bovine | 50 | 0/5 |
| 20 | + | bovine | 50 | 0/5 |
| Phase 4: digestion of products with different protease concentrations | – | bovine | 10 | 5/5 |
| 40 | – | bovine | 10 | 5/5 |
| 30 | – | bovine | 10 | 5/5 |
| Phase 5: specificity of the optimised conditions | – | bovine | 10 | 0/20³ |

¹Number of amplification reactions producing protease-resistant PrPSc/total number of reactions
²These were relatively weak signals on western blots
³Control sPMCA reactions not containing any PrPSc seed
previously reported. 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 10^6 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
sensitive, PrP\textsuperscript{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\textsuperscript{Sc} seed did not produce any detectable PrP\textsuperscript{Sc} triplet, only a single undigested PrP\textsuperscript{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\textsuperscript{Sc} signal produced when digesting samples with 10 or 50 \(\mu\)g/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 \(\mu\)g/ml PK before western blot analysis and probing with anti-PrP antibody SHa31. Molecular weight markers are indicated.

**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               | 1/2                | 1/2               |
| $10^{-6}$                   | 2/2               | 2/2               | 1/2                | 1/2               |
| $10^{-7}$                   | 2/2               | 2/2               | 1/2                | 1/2               |
| $10^{-8}$                   | 2/2               | 0/2               | 0/2                | 0/2               |
| $10^{-9}$                   | 0/2               | ND                | ND                 | ND                |
| $10^{-10}$                  | 0/2               | ND                | ND                 | ND                |
| $10^{-11}$                  | 0/2               | ND                | ND                 | ND                |
| $10^{-12}$                  | 0/2               | ND                | ND                 | ND                |

\(^a\) $10^{-2}$ dilution contains 100 \(\mu\)g of brain tissue and is the equivalent of using 1 \(\mu\)l of a 10% (w/v) brain homogenate as the seed in a 100 \(\mu\)l sPMCA reaction.

\(^b\) The sample was a pool of 10 bovine BSE isolates mixed in equal volumes.
differed in the molecular weight of the unglycosylated PrPSc 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 PrPSc 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 PrPSc and rely on the presence of relatively high levels of PrPSc in the caudal region of the brainstem. Such tests may not be sensitive enough to detect PrPSc 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 compared 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 average18 compared 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 PrPSc in mainly
older animals. The presence of relatively low levels of PrPSc in atypical BSE cases, compared with C-BSE, is likely for most age groups of cattle. Therefore the detection of PrPSc 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 PrPSc 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. 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.

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 and approximately 4 orders of magnitude more sensitive than commercial immunoassays. 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.
Masujin and coworkers propose a scheme to use QuIC to distinguish H-, L-, and C-BSE infections. 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 compared 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 ($\leq 10^{-5}$ 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 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. 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. sPMCA was performed basically as previously described 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 ultrasonicking water bath (model 4000; Misonix) at 37°C. Sonication 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 PrPSc 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 PrPSc 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 PrPSc when samples were digested at 10 and 50 μg/ml PK were determined using densitometry to determine total PrPSc signals as described above.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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