

Ruminant Prion Disease Detection and Characterisation Using Protein Misfolding Cyclic Amplification

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

September 2016

Abstract

Prion diseases or transmissible spongiform encephalopathies (TSE) are characterised by the accumulation of a misfolded conformer (PrP^{Sc}) of a host encoded protein (PrP^{C}). The misfolding event that leads to the formation PrP^{Sc} can be replicated in the *in vitro* amplification technique, protein misfolding cyclic amplification (PMCA). This thesis focuses on the application PMCA to study multiple aspects of prion misfolding in relation to ruminant prion diseases, specifically developing techniques to detect and characterise PrP^{Sc} in scrapie and BSE infections.

Utilising recombinant hamster PrP (rPrP) as substrate in PMCA, multiple genotypes of scrapie were successfully amplified in an attempt to describe a quantifiable technique applicable to a wide range of scrapie isolates. Observations of non-specific protease resistant rPrP formation was investigated with modifications to the PMCA methodology, which ultimately proved unsuccessful in reducing non-specific protease resistant rPrP. Using brain PrP^{C} as substrate, the quantitative PMCA technique was piloted with BSE to correlate *in vitro* replication efficiency with infectious titre in mouse bioassay, but no correlation was identified.

Atypical forms of BSE occur primarily in older cattle, are asymptomatic and thought to be spontaneous diseases. None the less, infection models in rodents and primates have identified the zoonotic potential of H-type and L-type BSE. Therefore PMCA methods were developed which were able to successfully amplify both atypical forms of BSE. In particular, sensitive detection and discrimination from classical BSE was demonstrated for H-type BSE, which has not previously been amplified in PMCA. H-type BSE could be detected in $1x10^{-12}$ g brain material and was discriminated from classical BSE by increased protease sensitivity, relatively high molecular weight and antibody reactivity.

Evidence exists for co-infection of TSE strains, yet scrapie and BSE co-infection in an ovine host remains unaddressed. To study the disease progression and tissue dissemination of co-infections a PMCA assay capable of specifically amplifying BSE PrP^{Sc} in the presence of excess scrapie was applied to artificially mixed brain homogenates containing BSE and scrapie, and compared to current statutory strain typing methods. The PMCA was found to have sensitivity and specificity of 100% in mixes containing 0.1% BSE and 99.9% scrapie brain material, which was more effective than conventional strain typing methods. The assay was then applied to the brain, spleen and lymph of scrapie and BSE experimental co-infections in two genotypes of sheep, and to animals which belonged to a flock with endemic natural scrapie and that also received experimental BSE infections. The PMCA data demonstrated that sheep with *PRNP* genotype ARQ/ARQ (at amino acid positions 134, 154 and 171) were resistant to BSE in a co-infection scenario. In sheep with *PRNP* genotype of VRQ/ARQ, mixed infections could occur,

and animals with scrapie PrP^{Sc} only in the brain could harbour BSE PrP^{Sc} in peripheral tissues. Co-infection was also possible in sheep with natural scrapie infections. The assay was compared to conventional testing methods of western blotting, PrP^{d} profiling and immunohistochemistry and displayed superior sensitivity in BSE detection.

PMCA amplification of bovine BSE isolates in ovine substrates identified several instances in which the molecular characteristics of the PrP^{Sc} was scrapie-like in terms of molecular weight, antibody reactivity and glycoform profile, and in some cases PrP^{Sc} characteristic of BSE could no longer be recovered. This occurred in a genotype specific manner, 'molecular switching' was only apparent in ovine substrate VRQ/VRQ in accordance with previous findings. These results raise the possibility of such an event occurring in *in vivo* ovine BSE infections and the zoonotic potential of these scrapie like conformers are yet to be fully addressed.

Publications

The following publications contain work from this thesis;

M.M. Simmons, M.J. Chaplin, C.M. Vickery, S. Simon, L. Davis, M. Denyer, R. Lockey, M.J. Stack, M.J. O'Connor, K. Bishop, K.C. Gough, B.C. Maddison, L. Thorne, and J. Spiropoulos. Does the Presence of Scrapie Affect the Ability of Current Statutory Discriminatory Tests To Detect the Presence of Bovine Spongiform Encephalopathy? *J. Clin. Microbiol.*, vol. 53, no. 8, pp. 2593–604, Aug. 2015

M.J. O'Connor, K. Bishop, R.G. Workman, B.C. Maddison, K.C. Gough. *In vitro* amplification of H-type atypical bovine spongiform encephalopathy by protein misfolding cyclic amplification. Prion 11, 54–64. Jan. 2017

“Can’t Pick Up No Crown,

Holding What’s Holding You Down”

-Killer Mike, Run the Jewels.

Acknowledgements

I would firstly like to thank my supervisors, Dr Kevin Gough and Dr Ben Maddison for their support. Without their guidance, insight and unwavering patience with me I would not have got here.

I must also thank members of ADAS biotechnology at the Nottingham school of veterinary medicine and science; Keith Bishop, Claire Baker, Jon Owen, and Helen Rees, for your guidance laboratory support but mostly for keeping me sane.

Thanks to all my friends at the University of Nottingham who have helped me throughout my time here, and I must express my deepest gratitude to Mr Robert Workman, without your superior musical tastes I may not have made it.

I would like to thank my supportive family for always being there and never doubting me. Finally I must thank Claire, for your belief, support and forever keeping me smiling.

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Chapter 1. Introduction

1.1 Introduction to transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are a group of infectious neurodegenerative diseases that affect mammalian species and are always fatal once contracted. Instances of TSEs have been documented in a range of mammalian species. In humans, TSEs include Creutzfeldt-Jakob disease (CJD,) GSS (Gerstmann-Straussler-Scheinker), fatal familial insomnia (FFI) and kuru (table 1.1). TSEs are also prevalent in wild and farmed animals, having both an economic and ecological impact (table 1.2). Scrapie which naturally affects sheep and goats has been documented since 1732 in the UK (McGowan, 1922). Bovine spongiform encephalopathy (BSE) is the only known zoonotic TSE and ingestion of infected bovine meat was responsible for cases of variant CJD in humans from the mid 1990s (Will et al., 1996). Wild and farmed deer and elk populations suffer from chronic wasting disease and transmissible mink encephalopathy was a problem in farmed mink.

TSEs by definition must be infectious, yet the aetiology is more complex as prion diseases can be acquired, genetic or sporadic. Acquired transmission involves either experimental introduction of PrP^{Sc} or natural infections. Natural infections occur primarily via oral ingestion of PrP^{Sc} in the diet, For example this is the case with BSE and kuru. In some animal diseases, environmental reservoirs of infectivity and direct animal-animal contact also play a role in acquired infections. Furthermore horizontal transmission *in utero* and via milk has been documented (Garza et al., 2011; Konold et al., 2013; Nalls et al., 2013). TSEs can be inherited via mutations in the PrP gene *PRNP* such as Creutzfeldt-Jakob disease and Gerstmann-Straussler-Scheinker disorder (Chesebro, 2003). These mutations are thought to increase the likelihood of a PrP^{C} molecule folding into a disease associated conformer. Sporadic disease occurs in cases where there are no familial links, mutations in *PRNP* or infectious source of PrP^{Sc}. Sporadic incidences of CJD are by far the most common and are the result of random PrP^{C} misfolding events.

Table 1.1. Summary table of human TSEs and the common transmission route for respective diseases.

Human TSEs	Common transmission route	First description[a]
Gerstmann-Sträussler-Scheinker disease (GSS)	Heritable mutations in *PRNP* gene	(Gerstmann et al., 1954)
Familial Creutzfeldt-Jakob disease (fCJD)	Heritable mutations in *PRNP* gene	(Creutzfeldt, 1920; Jakob, 1921; Spielmeyer, 1922)
Sporadic Creutzfeldt-Jakob disease s(CJD)	spontaneous misfolding event, without genetic or infectious source	(Creutzfeldt, 1920; Jakob, 1921; Spielmeyer, 1922)
Variant Creutzfeldt-Jakob disease (vCJD)	Oral exposure to exogenous prions	(Will et al., 1996)
Iatrogenic Creutzfeldt-Jakob disease (CJD)	Iatrogenic exposure to exogenous prions	(Duffy et al., 1974)
Fatal Familial Insomnia (FFI)	Heritable mutations in *PRNP* gene	(Lugaresi et al., 1986)
Kuru	Oral exposure to exogenous prions	(Hadlow, 1959)

a) The first description(s) of each TSE is referenced, some disease such as CJD were described multiple times independently, both references and a final reference drawing the two together are included in this case.

Table 1.2. A summary of the TSEs that affect animals.

Animal TSEs	Natural hosts[a]	Known susceptible species[b]	First description
Bovine Spongiform Encephalopathy	Cattle	Sheep, goat, mice, hamster, human, macaques, domestic cats, cheetahs, puma, ocelot, tiger, nyala greater kudu, Arabian oryx, eland, ankole cattle, gemsbok, scimitar-horned oryx and bison	(Wells et al., 1987)
Scrapie	Sheep and goat	Mice and hamster	(McGowan, 1922)
Chronic Wasting Disease	Deer, elk and moose	Mice and hamster	(Williams and Young, 1980)
Transmissible Mink Encephalopathy	Mink	Mice and hamster	(Marsh and Hadlow, 1992)

a) Natural hosts refer to the species in which respective TSEs originated and natural infections occur in.
b) Susceptible species are those that can contract the respective TSEs yet only in an experimental setting or through infection by contaminated feedstuffs. The first description of these TSEs are referenced in the final column.

TSEs are caused by and can be identified by the misfolding and subsequent aggregation of the host encoded cellular prion protein (PrP^C) (Prusiner, 1982). PrP^C is predominantly expressed in the central nervous system (CNS) and as such this is the primary location of the misfolding aggregation of the aberrant conformer of PrP^C, termed PrP^{Sc} (Benvegnù et al., 2010). Accumulation of PrP^{Sc} in the CNS and accompanying neurotoxicity develops into the characteristic plaque formation which is unique to TSEs and can be used to discriminate between different TSE infections by the specific spongiform vacuolation and PrP^{Sc} accumulation patterns they cause.

The misfolding of PrP^C to PrP^{Sc} is autocatalytic meaning that PrP^{Sc} has the potential to misfold PrP^C, providing there is no transmission barrier; it is this property that makes TSEs transmissible (Griffith, 1967; Prusiner, 1982). Infection of an individual with exogenous PrP^{Sc} may result in the misfolding of native PrP^C and therefore disease, providing that amino acid sequence homology allows for the PrP^C to be misfolded by that particular conformation of PrP^{Sc} (Collinge, 1999). The transmission barrier describes this phenomenon in which PrP^{Sc} and PrP^C must be compatible for disease progression and

infection to occur. This was first described as the "species barrier", as mice infected with sheep scrapie had very low attack rate and varied incubation times in primary passage but on secondary passage attack rates increased with consistent shorter incubation periods (Pattison, 1965). The susceptibility of PrP^{C} to misfolding by PrP^{Sc} is primarily a result of the secondary structure of PrP^{C} dictated by the amino acid sequence. This demonstration of the importance of primary structure over species associated co-factors led to the renaming of the species-barrier as the transmission barrier.

Transmissible spongiform encephalopathies share common clinical pathologies. These histopathological features of TSE infections include the characteristic spongiform degradation, loss of neurones, astrogliosis and microgliosis. The neurodegeneration that manifests as vacuolation can present as diffuse, concentrated or confluent vacuoles in the neuropil. Misfolded PrP^{Sc} accumulates in amyloidal plaques in the brain, but the frequency, size and structure of these aggregates vary depending on the strain of TSE causing the infection. The plaques can be concentrated as in those patients suffering from Kuru, florid as in vCJD cases or morphologically multicentric typical of GSS. The exact mechanism for neuronal death is not known yet the loss of neurones provokes gliosis. The distribution of these plaques can be located in diverse areas of the brain, which can then be used to create anatomical lesion profiles to discriminate between TSE strains.

It is the infectious nature of TSEs that distinguishes them from other protein misfolding disorders. Many other misfolded proteins have a role in disease pathology, and disease associated conformers of specific proteins can cause a range of other diseases including Parkinson's, Alzheimer's, Huntingdon's, frontotemporal dementia and type 2 diabetes (Soto et al., 2006). The cellular proteins that have the propensity to form these amyloidal diseases are diverse with varied functions and include tau, β-amyloid (Aβ), α-synuclein and islet amyloid polypeptide (Glenner and Wong, 1984; Grundke-Iqbal et al., 1986; Jaikaran and Clark, 2001; Trojanowski and Lee, 1998). These diseases are not proven to be infectious in natural settings, yet some evidence has been reported for the iatrogenic spread for some of these disorders. Aβ deposits have been found in the blood vessels, brain and pituitary gland of individuals, aged 36-51, suffering from iatrogenic CJD (iCJD) after receiving cadaveric growth hormone as children, yet these patients had no risk factors for early onset dementias (Jaunmuktane et al., 2015). Similar Aβ deposits have been found in iCJD patients that received dura mater grafts (Frontzek et al., 2016). Such deposits are early indicators of Alzheimer's disease which suggests that these individuals may have developed end stage Alzheimer's pathology as a result of these transplants, had they not first died of CJD (Jack et al., 2010). A growing body of work is beginning to indicate that there is 'cross talk' between these diseases, and misfolding of one aggregate-prone protein can begin the folding of another in heterologous seeds. Such heterologous seeds observed in human clinical disease include the presence of PrP^{Sc} in Aβ plaques in Alzheimer's affected brains, while

Aβ has been isolated from PrP^{Sc} deposits in CJD and GSS (Debatin et al., 2008; Ferrer et al., 2001; Miyazono et al., 1992). Together this can produce complicated disease pathologies in which several misfolded proteins are identified in one individual which blurs the boundaries between well-defined pathological diseases and highlights the similarities in these protein misfolding disorders.

1.2 Nature of the prion protein

The cellular prion protein is expressed in many diverse cell types in different tissues, and is predominantly expressed in the central nervous system, specifically in the neurones and glial cells of the brain (Fournier et al., 1998; Horiuchi et al., 1995; Piccardo et al., 1990). PrP^{C} is a conserved protein with homologues found in mammals, birds, reptiles, amphibians and fish (Gabriel et al., 1992; La Mendola and Rizzarelli, 2014). It is encoded by the *PRNP* gene and is well conserved within the *mammalia*. This level of conservation hints at an important role the prion protein, yet a definitive role of the protein is yet to be identified, while many putative roles have been theorised. PrP^{C} contains an octapeptide repeat located close to the N-terminus of the protein (Figure 1.1). This repeat readily binds divalent cations including, copper, manganese, zinc, iron and nickel (Brown et al., 2000; Brown et al., 1997). The binding of copper by PrP^{C} seems to have particular biochemical importance and strong evidence exists for PrP^{C} contribution to copper internalisation and metabolism. Knockout mice for PrP^{C} have reduced copper at synaptosomes and copper uptake into cells (Brown, 2003, 1999) while levels of PrP^{C} expression increase in response to exposure of cell lines to copper (Brown et al., 1997). Alteration of expression levels of PrP^{C} causes changes in proteins known to contribute to copper metabolism, suggesting a role for PrP^{C} in copper metabolism (Kralovicova et al., 2009). PrP has demonstrated superoxide dismutase (SOD) like activity suggesting that PrP^{C} may be part of the oxidative stress response and this activity was reliant on the octapeptide repeat binding copper. The SOD enzymatic activity was acquired through copper binding during the folding processing of nascent PrP^{C}, and crucially this activity is not conferred to mature PrP^{C} after exposure to copper (Brown et al., 1999). However research using recombinant PrP points towards a role regulating responses to oxidative stress rather than possessing direct SOD activity (Jones et al., 2005). The role of PrP^{C} in copper homeostasis and involvement in oxidative stress has been linked to angiogenesis and intrauterine development, which are negatively affected in PrP^{C} knockout mice ($PRNP^{(0/0)}$) (Alfaidy et al., 2013). PrP^{C} null mice also have altered sleep patterns including increased sleep fragmentation, implicating PrP^{C} as a contributing factor to the circadian rhythm and proliferation of the sleep state (Tobler et al., 1997, 1996). In addition, the development of epithelial cells in the intestine appears to be influenced by PrP^{C} expression, which controls in part the polarisation, differentiation and

proliferation of these cells (Morel et al., 2008). PrP^C has also been implicated in maintaining signal transductions of action potentials in neuronal cells via the internalisation of metal ions (Kretzschmar et al., 2001; Mouillet-Richard et al., 2000).

Despite observations such as altered sleep patterns, angiogenesis, copper metabolism and SOD activity in *PRNP*$^{0/0}$ mice, development of the knockout mice over entire lifespans show no significant dysfunctions, indicating redundancies in the functions of PrP^C and alternate genes with crossover function (Büeler et al., 1992).

The mammalian and avian genomic sequence encoding PrP^C is contained in one exon and codes for 253 amino acids in humans, 254 in mice, 256 in sheep, 264 in cattle (Figure 1.1). The protein has a mass of 30-39 kDa dependent of species sequence and is directed for processing in the endoplasmic reticulum by a 22 amino acid N-terminal signal peptide which is then cleaved in the ER (Turk et al., 1988). N-linked glycosylation occurs at position 180 and 196, leading to three possible glycosylation isoforms, unglycosylated, monoglycosylated and diglycosylated (Bolton et al., 1985). Post-translational modification also includes the removal of 23 C-terminal amino acids and subsequent addition of a glycosylphosphatidylinositol (GPI) anchor, which traffics and secures PrP^C to the outer layer of the plasma membrane (Stahl et al., 1987). As mentioned previously, PrP^C contains an octapeptide repeat, expansions of this repeat can increase sporadic TSE incidence in humans, and increase the rate of PrP^{Sc} formation, yet are not essential for TSE infections (Flechsig et al., 2000; Moore et al., 2006).

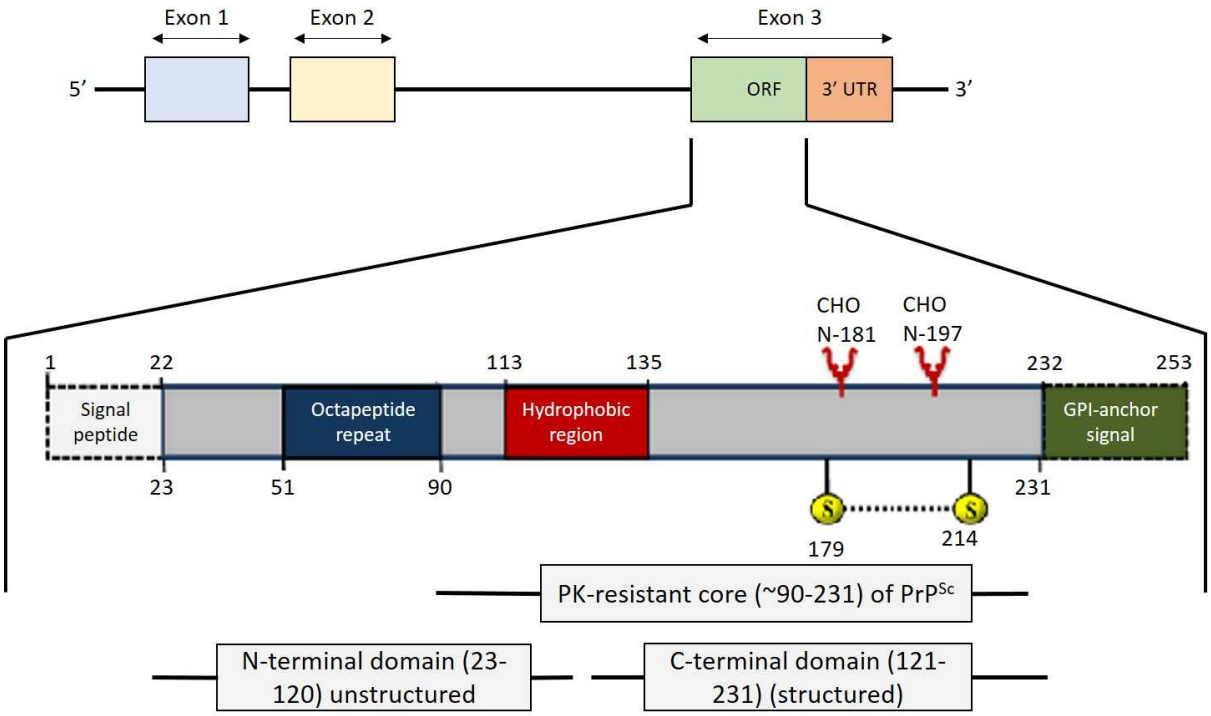


Figure 1.1. Structure of human *PRNP*. *PNRP* gene contains 3 exons but the only open reading frame is located in exon 3 which is translated into PrP. Immature PrP contains a signal peptide (codons 1 -22) which is cleaved as part of post-translational modification. The octapeptide repeat region can contain between 2 and 9 copies and is the region which binds divalent cations, such as copper. Increases in the octapeptide repeat copy number is associated with hereditary TSEs. There is a hydrophobic region of the protein (113 – 135) and a GPI-anchor signal (232 – 253). Two sites (181 and 197) are suitable for N-linked glycosylation, either both, one or neither of these sites can be glycosylated, giving 3 possible glycosylation isoforms of mature PrP. In mature PrP a disulphide bind is present between two cysteine amino acids (179 and 214). The PK-resistant core of PrP^{Sc} is highlighted (~90-231) and relatively unstructured and structured regions of the N and C-terminal domains. Figure adapted from (Acevedo-Morantes and Wille, 2014).

The secondary structure of PrP^{C} has been elucidated for many mammalian species (including human, mouse, hamster, cattle, goat and sheep) using nuclear magnetic resonance (NMR) and much of the structure is conserved between species. PrP^{C} comprises of 3 alpha-helical domains between residues 144-154, 175-193 and 200-219, an alpha-helical loop at 222-226 and two β-sheets at residues 128-131 and 161-164 (Riek et al., 1998, 1997).

The misfolding of PrP^{C} to PrP^{Sc} results in a drastic change in the secondary structure of the protein (modelled in figure 1.2). Using Fourier-transform infrared spectroscopy and circular dichroism, PrP^{C} was shown to have an α-helix content of 42% and very little β-sheet (3%). Upon misfolding the β-sheet content rose to 43% while α-helical content dropped to 21% (Pan et al., 1993). It is this change in conformational structure that confers the unique properties observed in PrP^{Sc}. It is theorised that upon misfolding the hydrophobic core of the protein is exposed, and this contributes to the

subsequent aggregation and resistance to proteinase digestion that is typical, but not a pre-requisite, of different PrP^{Sc} species. This exposure of hydrophobic residues and an increased propensity for self-association has prevented full NMR studies and other structural crystallography techniques being applied to PrP^{Sc}, as the PrP^{Sc} aggregates are not uniform making the crystal structures of poor quality.

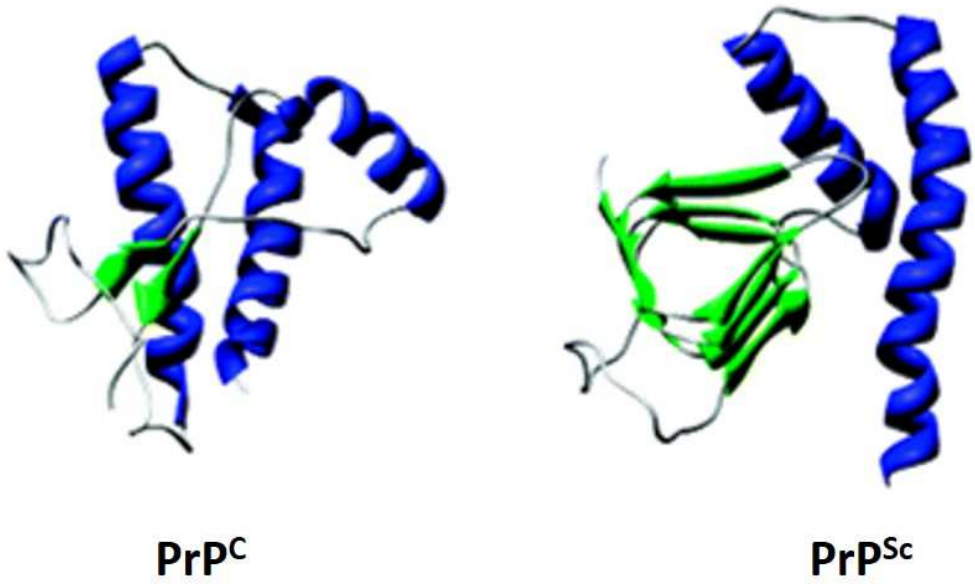


Figure 1.2. PrP^{C} (left) and PrP^{Sc} (right) modelled structures. The models demonstrate the change in secondary structure between the two isoforms. α-helical residues in blue, β-sheet residues in green. Adapted from (DeMarco et al., 2006)

The general model for the misfolding of other conformational diseases and TSEs are conserved. Misfolded proteins have a tendency to form structures rich in beta sheets, which organise into amyloidal aggregates. Two models exist to describe the formation of these amyloidal aggregates, the template assistance model and the nucleated polymerisation model (figure 1.3). In the template assistance model PrP^{Sc} is intrinsically more stable than PrP^{Sc}, interaction between monomers of the two isoforms leads to the formation of nascent PrP^{Sc} in an autocatalytic manner (figure 1.3) (Horiuchi et al. 1999; Tompa et al. 2002; Brundin et al. 2010). The nucleated polymerisation model predicts the formation of PrP^{Sc} in a similar process to nucleated polymerisation of crystals (Come et al. 1993; Jarrett et al. 1993). In this model PrP^{Sc} can be formed in a common misfolding event, but is extremely unstable and will revert to PrP^{C} or other unknown conformers, unless stabilised by interaction with amyloids (figure 1.3) (Horiuchi et al 1999; Tompa et al. 2002; Brundin et al. 2010). Alternatively in this model

misfolding is an autocatalytic process but can only be achieved when PrP^{C} reacts with PrP^{Sc} when present in a seed or amyloid (Horiuchi et al. 1999).

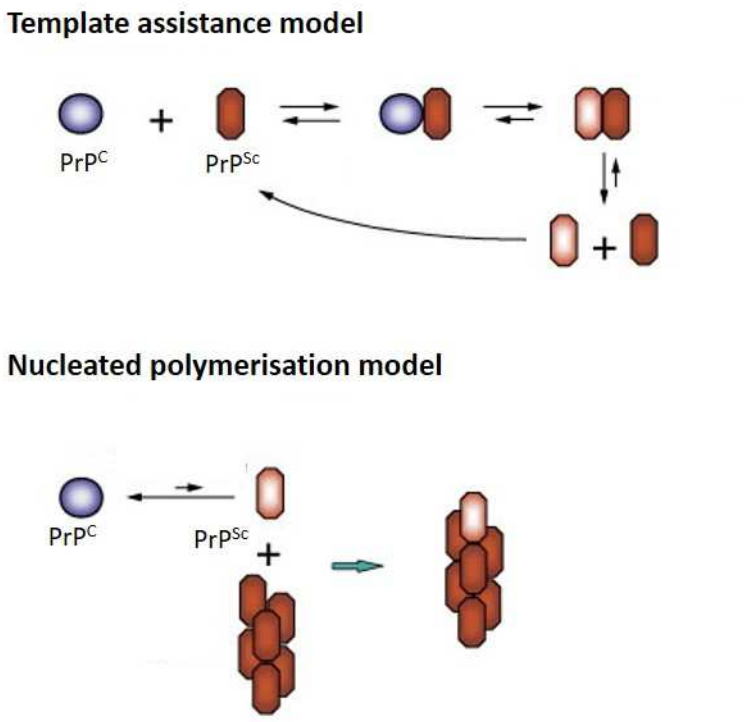


Figure 1.3. Template assistance model and nucleated polymerisation model. Two models exist that predict the process of PrP^{Sc} formation. In the template assistance model PrP^{Sc} is stable, and misfolding is an autocatalytic process occurring when PrP^{Sc} and PrP^{C} monomers interact. According to the nucleated polymerisation model, monomers of PrP^{Sc} are extremely unstable and revert back to PrP^{C} or other unknown conformers once formed, unless stabilised by interaction with amyloid polymers. In this model formation of PrP^{Sc} can occur either via random misfolding of PrP^{C} as depicted, or by PrP^{C} interaction with amyloid polymers directly. Figure adapted from (Horiuchi et al. 1999)

1.3 Construction of the Protein only Hypothesis

The concept of an infectious particle consisting predominantly, if not entirely, of a proteinaceous agent was met with some resistance when it was suggested by Prusiner in 1982 (Prusiner, 1982). No doubt this reaction was in part due to the fundamentally challenging nature of such a hypothesis, and the investment of other researchers in competing theories. However the idea of a protein only infectious model for scrapie was first suggested in a purely theoretical paper by Griffith in 1967 (Griffith, 1967).

Before the protein only hypothesis was founded, a multitude of explanations had been produced to explain the non-typical infections of sheep with scrapie that have been documented for more than

250 years. In 1954 Bjorn Sigurdsson described a group of viruses which he termed slow viruses and included the scrapie infection in this grouping. Although Sigurdsson successfully identified viral pathogens as the causative agents for slow infections such as visna, his assertion of scrapie as a similar slow viral infection set the course for the assumption of prions as viral infections and part of the subsequent resistance to the protein only hypothesis (Sigurdsson and Palsson, 1958).

In 1959 William Hadlow made a link between scrapie and kuru, an infectious neurodegenerative disease which was transmitted by the ritualistic cannibalistic practises of the Fore tribe of Papua New Guinea (Hadlow, 1959). Hadlow noted the similarity in pathogenesis of the two diseases, in particular that the drastic spongiform degradation was a hallmark of both diseases. The connection to a similar human condition and therefore implications of a possible zoonosis generated much interest in the scrapie agent and the theory of possible cross species infections was further supported by the experimental transmission of kuru and familial CJD to chimpanzees (Gadjusek et al. 1966; Gibbs et al. 1968).

Despite this renewed interest in the scrapie agent, no successful attempt could be made to isolate the scrapie virus, but discoveries about the nature of the infectious agent led to a wide-ranging hypothesis for a possible non-classical infectious agent. The nature of the scrapie agent, which was capable of transmitting to rodents while demonstrating strain characteristics, as well as being highly resistant to degradation and extremely difficult to isolate led to various hypotheses to explain these findings. Such hypotheses included the agent consisting of a small DNA virus, an “unconventional virus”, a viroid, a complex of carbohydrate molecules such as polysaccharides or oligosaccharides, a nucleoprotein complex, nucleic acid enclosed in polysaccharide and an infectious protein (Kimberlin et al. 1967; Gajdusek 1977; Diener 1972; Gibbons et al. 1967; Latarjet et al. 1970; Narang 1974; Prusiner 1982).

The majority of these novel theories to explain the scrapie agent were based on observations inconsistent with a virus or virus like particle containing nucleic acid, as traditional techniques capable of destroying nucleic acid had little effect on scrapie infectivity. For example the scrapie agent was stable in a wide range of pH, between 2 and 10.5, some of which would denature DNA (Mould et al., 1965). The scrapie agent was also completely resistant to both DNA and RNA nucleases, ultra-violet radiation and divalent cation hydrolysis (Prusiner et al. 1980; Alper 1970; Latarjet et al. 1970; Prusiner 1982). The maintenance of scrapie infectivity after such treatment could potentially be explained by a phenomenally protective protein coat, however UV resistance to that extent indicated a genome smaller than that required for replicative purposes. Furthermore the UV range most effective against scrapie did not align with the spectra that interacts with nucleic acids (Alper, 1970). Scrapie was also proven to be resistant to the psoralen photoreaction and chemical modification by hydroxylamine which combined inactivated all known viral pathogens (Bellinger-Kawahara et al., 1987; McKinley et

al., 1981). The scrapie agent also displayed extreme resistance to thermal inactivation by dry heating or autoclaving and to formaldehyde treatment, which led to the accidental infection of 1500 sheep by scrapie infected vaccinations (Brown et al., 1982). The use of heat and formaldehyde as a combination treatment also failed to have any significant impact on scrapie infectivity (Brown et al., 1990). Phenol extractions were also used to separate the nucleic acid and protein components yet failed to couple infectivity to the nucleic acids as would be expected for a virus or viroid (Ward et al., 1974).

While little evidence was found for a viral pathogen as an explanation for the properties of the scrapie agent, protein was found to be crucially important. Precipitation of proteins showed that scrapie infectivity co-purified with a particular protein which proved to be extremely hydrophobic (Prusiner et al. 1981). Scrapie infectivity was shown to be sensitive to proteinases and protein chemical modification by carbethoxylation (Prusiner et al. 1981). Ionic detergents, chaotrophic salts and urea all of which target the proteinaceous content of scrapie infected material proved effective at degrading the scrapie agent. While phenol extractions also showed that the infectious components of scrapie resided in the proteinaceous fractions (Prusiner et al. 1981; Prusiner et al. 1980; Prusiner et al. 1981).

Rod shaped particles were observed in association with infectivity which were described as protein aggregates (Prusiner et al., 1983). Evidence that these aggregates consisted of misfolded protein instead of the capsid of an elusive virus included the fact that these rods were not uniform (Prusiner et al., 1983). Differential centrifugation demonstrated that the infected scrapie agent varied in density and size unlike uniformly structured viral particles (Brown et al., 1978; Prusiner et al., 1978). Furthermore, no viral nucleic acid was identified in association with these structures, despite the fact that the detection techniques could identify one tobacco mosaic virus virion per ID_{50} of the scrapie agent (Gabizon et al., 1987).

The amassed evidence that highlighted the extreme resistance of scrapie to nucleic acid degrading agents and techniques but sensitivity to protein degrading treatments led Stanley Prusiner to develop the term prion (proteinaceous infections). This became the bedrock for the protein-only hypothesis that is now the scientifically accepted theory regarding the nature of transmissible spongiform encephalopathies (Prusiner, 1982). The hypothesis states that the infectious particle of TSEs consists of predominantly, if not entirely, of a misfolded conformer of the encoded PrP protein.

1.4 Transmissible Spongiform Encephalopathies

1.4.1 Human TSEs

Human transmissible spongiform encephalopathies include Cruetzfeldt-Jacob disease, fatal familial insomnia, kuru and Gerstmann-Sträussler-Scheinker syndrome. CJD cases can be sporadic, familial, or acquired. Sporadic CJD (sCJD) is the most common form of CJD affecting up to 2 people per million annually (Imran et al. 2011). There is no gender bias of infected individuals with average age of onset between 55 and 60 years. The clinical symptoms include progressive dementia, cerebellar dysfunction affecting locomotion, coordination, speech and communication. This progresses to the development of tremors, and dementia causing confusion, depression and personality changes. The cause of sCJD is not well understood, it is not caused by mutations in the *PRNP* gene or known exposure to sources of infectious prions. Higher risks of sCJD have been associated with *PRNP* genotypes with homozygosity for methionine or valine at codon 129 (Palmer et al., 1991). It has been proposed that sCJD is caused by random misfolding events that generate PrP^{Sc}, yet such a hypothesis remains difficult to prove, though spontaneous generation of prions *in vitro* have been documented widely. Other explanations include somatic mutations in PrP.

Familial CJD (fCJD) is caused by various mutations in the *PRNP* and was first identified in the 1920s and accounts for 5-15% of CJD cases in humans. Point mutations cause many of the mutations in the *PRNP* gene, however duplications of the octapeptide repeats of 2 copies and 5-9 copies and insertions have also been linked to familial cases of CJD. While these mutations are the cause of fCJD, other host and environmental factors are believed to contribute to various aspects of disease, as members of the same family with the same mutation display subtly different symptoms such as age of onset. Furthermore, certain mutations such as Lysine at residue 200 does not exhibit 100% penetrance as is the case with other disease associated changes to the PrP sequence. Whilst the Lys 200 mutation penetrance indicates that other host or environmental factors also contribute to disease progression, the identity or exact role of these factors are unknown (Goldfarb et al., 1991). fCJD has clinical symptoms typical of TSEs including progressive dementia, ataxia, spongiosis and myoclonus.

fCJD shares similarities with fatal familial insomnia (FFI). The substitution of asparagine for aspartic acid at position 178 in *PRNP* is linked to both familial CJD and FFI incidence, but are separated by a single polymorphisms at codon 129. Methionine at codon 129 is associated with FFI, whereas valine at this position is associated with CJD (Goldfarb et al., 1992). Symptoms of FFI are also closely related to familial CJD, with dementia, ataxia and myoclonus, but FFI displays reduced spongiosis, increased atrophy and insomnia.

Gerstmann-Straussler-Scheinker (GSS) syndrome was first described in 1936 and is estimated to affect 1 in 20 million people. It is characterised by the presence of numerous amyloidal plaques in the central nervous system, ataxia, locomotion difficulties and dementia, though a wide range of other clinical symptoms are also observed in patients depending on specific mutations in the *PRNP* gene. GSS is caused by autosomal dominant mutations, primarily involving codon 102, though can be caused my mutation in at least 5 other amino acid positions causing distinct disease progressions. Disease caused by the mutation at codon 102 has an average age of onset of 48 years, yet disease progression is relatively slow lasting on average 5 years. In contrast a mutation at 117 causes disease with diminished levels of ataxia and an average onset age of 38, while variation at position 198 is associated with clinical onset averaging at 52 years (Belay, 1999).

Variant CJD (vCJD) was identified in the UK in 1996 after 10 patients in the previous 2 years had displayed Creutzfeldt-Jakob disease with sporadic symptoms. Since its discovery over 200 cases of variant CJD have been confirmed. The age of onset of clinical disease in these patients was extremely low ranging between 16 and 72 years with the average age for this group being 28 years compared to 65 for sporadic CJD patients. An early age of onset not associated with classical CJD was accompanied by prolonged disease durations. sCJD disease durations is between 2.5 and 6.5 months, whereas for vCJD it is 7.5 and 22 months post diagnosis. Furthermore, significant behavioural changes occurred at disease presentation as well as early ataxia, another symptom not often associated with sCJD, while the development of progressive dementia was delayed compared to sCJD. The neuropathological features of vCJD include spongiform change and neuronal loss in the basal ganglia and thalamus and also clustered in the cerebellum and cerebrum. The PrP^{Sc} plaques found in the cerebrum and cerebellum areas were not typical of sCJD and were also far more dispersed throughout the brain regions affected than for sCJD. The centre of the plaques were dense and stained strongly for eosin which is more similar to plaques associated with Kuru rather than sCJD. The plaques were also encompassed by spongiform change, which is not seen with sCJD patients. (Belay, 1999; Will et al., 1996).

The development of variant CJD was linked to BSE when it was first described, as the increase in CJD surveillance was in response to fears of the zoonotic potential of BSE. After this speculation the link was proven in part by evidence that PrP^{Sc} of vCJD closely resembled that of BSE from various species of infected animals, rather than the PrP^{Sc} isolated from the patients with sporadic CJD (Collinge et al., 1996). Macaques infected with BSE displayed symptoms strikingly similar to humans suffering from vCJD, including behavioural changes as first clinical signs followed by ataxia and neuropathology with florid kuru-like plaques (Lasmézas et al., 1996). Furthermore, mice inoculated with PrP^{Sc} from

confirmed cases of BSE or vCJD were indistinguishable from each other expressing identical lesion profiles, plaque morphologies and incubation periods (Bruce et al., 1997)

1.4.2 Scrapie

Scrapie is the archetypal TSE and has been documented for over 250 years; the first documentation is credited to 1732. Symptoms of scrapie in sheep include irritation of the skin, and loss of coat due to scratching, which is thought to have given rise to the name scrapie. Scrapie infected sheep also suffer from other classical TSE symptoms such as locomotive problems, ataxia and vacuoles in the brain (Chesebro, 2003; Plummer, 1945). Scrapie does not exist as one discreet entity but consists of diverse strains which cause subtly different disease. The phenomenon of scrapie strains was first identified when several different disease phenotypes were observed in goats infected with scrapie, presenting disease of either drowsy or scratching subtypes (Pattison et al. 1961). Despite the existence of various strains of scrapie none has ever been shown to be a likely zoonotic agent.

In 1970 an isolate of scrapie was identified in the UK named CH1641 as it had a unique incubation period in ovine transmission studies in Cheviot sheep (Foster et al. 1988). After the BSE epidemic the molecular properties of the CH1641 PrP^{Sc} were analysed alongside other strains of scrapie and BSE. CH1641 was indistinguishable from BSE in terms of its molecular weight and electrophoretic properties (Hope et al., 1999). However CH1641 could be differentiated from BSE based on its glycosylation profile. Scrapie strains including CH1641 have relatively equal proportions of di-glycosylated and mono-glycosylated PrP^{Sc} whereas BSE favours the di-glycosylated isoform of PrP^{Sc} (Hope et al., 1999; Stack et al., 2002).

Atypical scrapie, is also known as Nor98 due to its primary isolation in Norway in 1998 but has since been isolated from many European countries. Atypical scrapie differs from classical strains of scrapie in its disease pathology and both biochemical and molecular properties of PrP^{Sc} (Benestad et al., 2003). Atypical scrapie cases have predominantly been identified due to routine surveillance procedures in apparently healthy animals and statutory testing of fallen stock, which implies atypical scrapie may present less severe clinical symptoms or be subclinical (Benestad et al., 2008). PrP^{Sc} deposition in the brains predominantly occurs in the cerebellum and cortex with atypical scrapie and this is at low levels, whereas classical scrapie and ovine BSE deposits relatively high levels of PrP^{Sc} and mostly in the brain stem (Benestad et al., 2003). Furthermore, classical scrapie spreads to the animals lymphoreticular system before reaching the brain, but atypical scrapie shows a limited ability to replicate in the peripheries (Benestad et al., 2003; Nentwig et al., 2007). Nor98 PrP^{Sc} is more sensitive to proteases than the PrP^{Sc} of classical scrapie strains. In addition, a small molecular weight protein (10-12 kDa) is

found in Nor98 samples after protease degradation and SDS-PAGE electrophoresis in addition to the three larger proteins found in classical scrapie (Benestad et al., 2003; Gavier-Widen et al., 2004).
Atypical scrapie has infected animals with a wide range of genotypes for *PRNP*, including those that are most resistant to classical scrapie infections (including ARQ/ARR and ARR/ARR). Therefore, sheep that were bred for classical scrapie resistant *PRNP* genotypes have been associated with Nor98 infections (Baron et al., 2007). Atypical scrapie is thought to be a sporadic TSE infection similar to sporadic prion disease found in humans, which may account for the atypical disease progression, biochemical, and molecular differences observed with classical scrapie.

1.4.2.1 Ovine genetics of scrapie

Within the *PRNP* gene there are 15 documented codon polymorphisms in the structured C-terminal region of the protein, however only 3 positions have demonstrated significant effects on the susceptibility to scrapie infection in sheep (Baylis and Goldmann, 2004). These polymorphisms are located at amino acid positions 136, 154 and 171 and changes from alanine (A) to valine (V), from histidine (H) to arginine (R) and from arginine (R) to glutamine (Q) or histidine (H) at these positions respectively are associated with increased susceptibility to scrapie (Baylis and Goldmann, 2004). Of the possible combinations of these alleles only 5 occur frequently which are $V_{136}R_{154}Q_{171}$ (VRQ), $A_{136}R_{154}Q_{171}$ (ARQ), $A_{136}R_{154}H_{171}$ (ARH), $A_{136}R_{154}R_{171}$ (ARR) and $A_{136}H_{154}Q_{171}$ (AHQ). To breed scrapie resistant genotypes into the UK and eradicate those with high susceptibility the most common genotypes were divided into 5 risk categories (table 1.3) (Zou and Gambetti, 2013). The susceptibility of each genotype has been demonstrated by analysing the number of reported cases per annum per million sheep for each genotype in the UK (Baylis et al., 2004). Using this method the most susceptible genotype was found to be VRQ/VRQ followed by ARH/VRQ, ARQ/VRQ, and then ARQ/ARQ, while the most resistant genotypes were ARR/ARR, ARR/ARH, and AHQ/ARH.
With regards to genotype effect on BSE infection extensive studies have found that all sheep genotypes tested for polymorphisms at positions 136, 154 and 171 produce positive disease. However further substitutions of leucine to phenylalanine at codon 141 and methionine to threonine at codon 112 are associated with reduced infection rates (Houston et al., 2015).
In 2001 in the UK the National Scrapie Plan (NSP) was introduced with the aims to reduce incidence of scrapie in the UK sheep population and to shield consumers of UK sheep from BSE exposure (Ortiz-Pelaez et al., 2014). Part of the NSP included the Ram Genotyping Program which genotyped around 3 million sheep before its termination in 2009. The NSP sought to reduce the frequency of the susceptible VRQ allele while simultaneously increasing the population of the resistant ARR allele

within the UK national flock. Over this period there has been a 90% reduction in the number of classical scrapie cases reported in the UK, although the contribution of the NSP to this reduction is not clear (Arnold and Ortiz-Pelaez, 2014). Over the same period the incidence of atypical scrapie in the UK has remained constant which is in accord with cases of atypical scrapie in ARR/ARR sheep and observations that the ARR genotype has the highest frequency of atypical scrapie cases (Kittelberger et al., 2010).

The first effort to clone and sequence *PRNP* in sheep in 1990 identified the arginine and glutamine polymorphism at position 171 (Goldmann et al., 1990). This resistance of Q_{171} was identified by further study which also highlighted the role of the alanine to valine polymorphism at amino acid position 136 as a potential codon with high implications for scrapie susceptibility in sheep (Goldmann et al., 1991). Furthermore studies using denaturing gradient gel electrophoresis identified position a substitution at codon 154 from arginine to histidine in sheep more likely to be infected by scrapie (Laplanche et al., 1993). Finally additional variants at position 171 coding histidine were isolated from a flock of sheep with an endemic scrapie infection and associated with lower infection rates compared to glutamine at the same position (Clouscard et al., 1995).

Table 1.3 Susceptibility of ovine *PRNP* alleles to scrapie

Allele risk group[a]	**Genotype[b]**
Highest susceptibility	VRQ/VRQ
High susceptibility	ARQ/VRQ
	ARH/VRQ
	AHQ/VRQ
	ARR/VRQ
Low resistance	ARQ/ARQ
	ARH/ARQ
	ARH/ARH
	AHQ/ARQ
	AHQ/ARH
	AHQ/AHQ
Resistant	ARR/ARQ
	ARR/ARH
	ARR/AHQ
Highest Resistance	ARR/ARR

a) Risk group according to susceptibility to scrapie infection
b) Genotype of *PRNP* at amino acid position 136, 154 and 171

1.4.3 Bovine spongiform encephalopathy

Bovine spongiform encephalopathy was responsible for an epidemic in UK cattle in the late 1980s and early 1990s. It is believed the disease agent was disseminated in feed due to changes in the processing of meat and bone meal (MBM) a commonly used feed stuff consisting of animal product, including cattle and sheep. To increase the fat content in MBM the hydrocarbon solvent extraction steps were no longer used in the processing of MBM towards the end of the 1970s. This is thought to be one factor that may have allowed BSE prions to persist in the MBM which was fed to cattle. The cattle which were fed MBM containing BSE PrP^{Sc} were infected and in turn used to create MBM that was then used as feed for yet more cattle. This process recycled BSE into the MBM until an estimated 2 million animals became infected. In 1988 feeding MBM to animals was banned in the UK, however during that time infected cattle made it into the food chain, especially as the incubation time for BSE in cattle to clinical disease is on average 5 years and animals were slaughtered at around 3 years of age for human consumption. (Prusiner 1998; Collinge et al. 2007; Chesebro 2003; Doherr 2007). The origin of the BSE prion is still unknown but one theory is that scrapie crossed the transmission barrier to infect cattle through the MBM, as ovine material was included in MBM. Alternatively, a hypothetical spontaneous bovine TSE could have occurred and been disseminated via MBM (Doherr, 2007; Group, 1998). An unusual trait of the BSE agent is that it has crossed the transmission barrier to infect a wide range of species via PrP^{Sc} containing bovine material in feed/food. As well as man, other affected species included BSE in exotic ungulates and goats, and feline spongiform encephalopathy in captive cheetahs, pumas and in both wild and domestic cats (Summary in table 1.2) (Chesebro 2003; Prusiner 1998; Cunningham et al. 2004; Eiden et al. 2010; Sigurdson et al 2003).

To date two forms of atypical BSE has been described which are the bovine amyloidotic spongiform encephalopathy (BASE) or L-type BSE and H-type BSE. L-type BSE was identified in Italy in 2003 in 2 cattle and has PrP^{Sc} with a lower molecular mass than classical BSE. L-type BSE also shows a preference for monoglycosylated PrP^{Sc} and when used to infect bovinised transgenic mice displays different incubation times and patterns of spongiform change to classical BSE (Capobianco et al., 2007; Casalone et al., 2004). H-type BSE was first described in France in 3 cattle, each over the age of 24 months. H-type BSE causes a different legion profile in cattle and other mammals it infects, compared to classical BSE. It was also noted that very little PrP^{Sc} was present in the brain stem (an area where PrP^{Sc} is predominantly deposited in classical BSE infections). The H-type BSE unglysocylated PrP^{Sc} has a higher molecular weight than classical BSE (a difference between 0.7 and 1.3 kDa). Due to this larger size of protease-resistant PrP^{Sc}, H-type BSE also reacts with antibodies to the N-terminal region of PrP (e.g. P4) that do not bind strongly with classical BSE. Furthermore, the H-type BSE PrP^{Sc} shows a higher percentage of monoglycosylated isoform compared to classical BSE (Biacabe et al., 2007, 2004).

It is theorised that both H-type and L-type BSE may represent sporadic TSE disease similar to those seen in humans, this would explain the extremely low infection rates observed in these two TSE strains and the tendency to infect older cattle compared to classical BSE (Biacabe et al., 2004).

1.4.4 Chronic wasting disease

Chronic wasting disease infects deer and elk and has been documented in US farmed animals from the 1940s but was recognised in 1978 as a TSE in captive mule deer (Williams and Young, 1980). A wide range of deer and elk species are natural hosts for CWD prions, including mule deer, white-tailed deer, black-tailed deer, Rocky Mountain elk and Shira's Moose, and experimentally red deer and reindeer are also susceptible (Imran et al. 2011). CWD infections can proceed through horizontal transmission, and through contaminated environments. PrP^{Sc} can be shed into the environment through faeces, urine, saliva, blood, and decomposing carcasses. In CWD PrP^{Sc} is deposited widely throughout the central nervous system, and also large deposits accumulate in the peripheries such as tonsils, lymph nodes, gastrointestinal tract and skeletal muscle (Haley et al., 2009a; Henderson et al., 2013; Sigurdson, 2008). Cases of CWD can be found in farmed and wild animal populations in the USA, Canada and South Korea, though widespread testing and surveillance has yet to be implemented outside of these countries (Sigurdson, 2008).

1.4.5 Transmissible mink encephalopathy

Transmissible mink encephalopathy (TME) has been documented in farmed mink in North America, Europe and Asia. TME was thought to have arisen from a contaminated feed stuff. Upon passage onto Syrian hamsters, TME has been shown to produce two distinct disease phenotypes and pathologies, termed hyper (HY) and drowsy (DR). Hamsters infected with the HY strain of TME suffer cerebellar ataxia and hyper excitability, whereas DR infected animals develop progressive lethargy. These strains also have different incubation periods, with HY strain killing host significantly quicker (average 65 days) than DR (average 168 days). Furthermore, the strains have different susceptible hosts, DR retains the ability to cause disease in mink, but HY is no longer able to cause TME in mink (R. A. Bessen and Marsh, 1992; Bessen and Marsh, 1992). The difference in these two strains of TME are thought to be the results of distinct misfolded conformers of PrP^{Sc}. HY and DR PrP^{Sc} show different physical and biochemical properties, such as sedimentation rate, protease sensitivity and molecular weight in SDS-PAGE electrophoresis. Ultracentrifugation and detergent extraction have demonstrated that DR is soluble and HY insoluble. While these differences are thought to be a product of different PrP^{Sc}

conformations it is important to note that both of these strains were isolated from Syrian hamsters with the same *PRNP* genotype; as such, biochemical or conformational differences are not a result of misfolding a different substrate or PrP^{C} primary sequence (Bartz et al., 2000; Bessen and Marsh, 1992; Bessen and Marsh, 1994; Bessen and Marsh, 1992).

1.5 Mixed infections

Multiple strains of the same TSE agents can be present in the same host at the same time.

Evidence for this included the isolation of hyper and drowsy strains of TME identified in the same isolate of the Stetsonville Wisconsin outbreak agent when passaged through hamsters, and these strains have different biochemical and clinical properties (Bessen and Marsh, 1992). A similar phenomenon has been observed with scrapie; two strains, ME7 and 87V, were identified from the same ovine scrapie sample when they were analysed in wild-type mouse bioassay (Thackray et al., 2012). While these examples in principle could argue for the existence of mixed TSE infections, neither study definitively demonstrated the presence of both strains in the original samples. It is conceivable that one of the strains (or both) was the product of "strain mutation" of the original inoculum as a result of crossing the transmission barrier. In this scenario a mixed infection may not have been present in the original samples used in bioassay but at least one strain was a *de novo* generation. However, natural atypical and classical scrapie strains have been isolated from the same ovine host in Italy (Mazza et al., 2010). As described, the pathology of atypical and classical scrapie is very different, in atypical scrapie PrP^{Sc} is primarily deposited in the cerebellar and cerebral cortex whereas these deposits occur most frequently in the brainstem of sheep infected with classical scrapie (Benestad et al., 2003). The brain regions in which PrP^{Sc} was deposited and other pathological signs indicated that both classical and atypical scrapie disease was present in the sheep. The PrP^{Sc} detected by western blot displayed the three glycoform profile associated with classical scrapie but also contained a smaller 10-12 kDa Nor98 band depending on which brain region the protein was isolated from. Furthermore, classical scrapie disseminates in the LRS whereas atypical scrapie does not. PrP^{Sc} was found in the LRS of this sheep but did not contain the small atypical indicative band on western blotting. In addition, a recent study of archived ovine samples of ~1850 healthy and scrapie infected individuals dating back to the 1960s identified 6 cases of atypical scrapie, 2 of these occurred in sheep during co-infection with classical scrapie (González et al., 2015). Several experimental models have been used to analyse scrapie and BSE mixed infections *in vivo*. Mice bioassay studies found that in mixed infections, scrapie PrP^{Sc} species could predominate and mask the presence of BSE PrP^{Sc} which may be present in lower titres, in such instances all bioassay results, lesion profiling, attack rates and incubation times indicate

a scrapie only infection. This appears to hold true when using mouse adapted BSE and scrapie strains in wildtype mice, or ovine adapted BSE and scrapie in ovine transgenic mice and even when initial doses of BSE were much higher than those of scrapie (Baron and Biacabe, 2001; Corda et al., 2015).

1.6 Prion amplification *in vitro*

1.6.1 History of *in vitro* prion replication

The wealth of *in vitro* amplification assays that are now available are based upon the initial proof of direct misfolding of PrP^{C} by interaction with PrP^{Sc} (Kocisko et al., 1994). Recombinant S^{35}-labelled PrP^{C} was shown to misfold into a protease resistant PrP^{Sc} conformer only in the presence of previously generated PrP^{Sc}. Furthermore, this ability of PrP^{Sc} to misfold PrP^{C} was hindered by denaturation of PrP^{Sc} with guanidine hydrochloride implying that the specific conformational shape of PrP^{Sc} plays a role in this conversion. In addition, the ability of PrP^{Sc} to misfold PrP^{C} into nascent PrP^{Sc} was shown to be dependent on the genotype of PrP^{C}, in much the same way transmission of TSEs in bioassays is host-genotype dependent (Raymond et al., 1997).

These observations were built upon to a produce a prion amplification process known as protein misfolding cyclic amplification (PMCA) in 2001 by Soto and colleagues (Saborio et al., 2001). In PMCA a small amount of seed (PrP^{Sc}) is incubated with an excess amount of substrate (PrP^{C}, usually within a brain homogenate) and the misfolding of this PrP^{C} into PrP^{Sc} by interaction with the seed proceeds during repeat cycles of sonication and incubation (figure 1.4). As the seed misfolds the substrate, it accumulates in amyloid fibrils or aggregates during the incubation periods of PMCA (Kocisko et al., 1994; Saborio et al., 2001). Sonication then breaks up these aggregates producing a greater number of aggregates or nucleation points (Saborio et al., 2001; Soto et al., 2002) and thus more available PrP^{Sc} to interact with and misfold PrP^{Sc}. By cycling 1 hour incubation periods at 37^{o}C followed by five 1 second bursts of sonication for up to 40 cycles, Soto and colleagues showed this produced much more newly folded PrP^{Sc} than previously documented for the *in vitro* replication of prions. Soto and colleagues improved the misfolding efficiency of the seed by the introduction of repeated, automated sonication cycles. This improved method used microsonicators with inbuilt water baths which could be set to deliver desired sonication periods at various power outputs. In addition to automation, PMCA rounds were extended by using PMCA products to seed new PMCA substrate after 24 hours, greatly improving the sensitivity of the method, this process became known as serial PMCA (sPMCA) (Bieschke et al., 2004). Protein misfolding by PMCA and sPMCA was much quicker than other *in vitro* approaches

making it an attractive method for studying many aspects of prion replication and prion diseases (Castilla et al., 2005; Saborio et al., 2001; Weber et al., 2007).

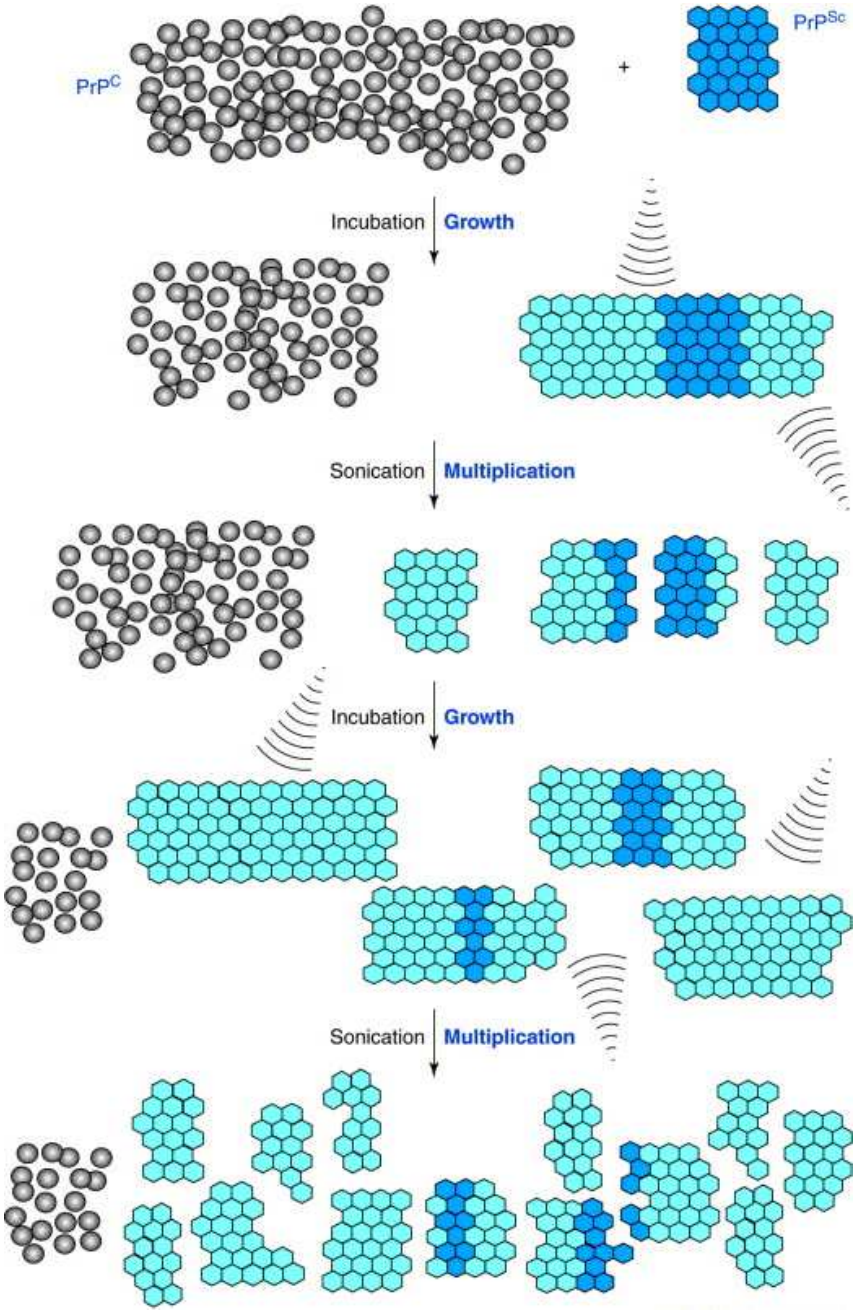


Figure 1.4. **Protein misfolding cyclic amplification.** The substrate for conversion, PrP^{C} (grey) is incubated in excess with prion seed, PrP^{Sc} (dark blue). During incubation PrP^{C} is misfolded into PrP^{Sc} in association with already formed PrP^{Sc} aggregates. The newly misfolded PrP^{Sc} is coloured light blue. The aggregates are broken up by sonication into a greater number of smaller aggregates. Further incubation sees these smaller fragments act as template for more protein misfolding, increasing efficiency. Again, the aggregates that are produced are fractured into smaller fragments by sonication

followed by incubation. Incubation and sonication periods are cycled to generate large amounts of newly generated PrP^{Sc} from small amounts of seed. Figure adapted from (Soto et al., 2002)

After the inception of PMCA as a tool for studying prion diseases, some investigators doubted the output was a result of a cyclical misfolding event, arguing products of PMCA were merely leftover seed PrP^{Sc} or a separate entity all together. These arguments were challenged by observations that infectious PrP^{Sc} could be detected by PMCA after a 10,000 fold dilution of initial infected brain homogenate; estimated to represent a single infectious unit made up of ~26 molecules of PrP^{Sc} (Saá et al., 2006a). Additional studies showed that PrP^{Sc} can be detected in PMCA products following a dilution of 10^{-55} of the scrapie source, yet only a dilution of 10^{-14} would be required to lose the initial PrP^{Sc} molecules present in the seed (Castilla et al., 2005). In addition, PrP^{Sc} can be generated from recombinant substrate only; refuting contributions of hypothetical accessory proteins or co-factors that may be present within substrates such as brain homogenates (Geoghegan et al., 2009). These studies provide comprehensive proof that the PMCA products are newly generated PrP^{Sc} and not seed derived PrP^{Sc} and that the PMCA process is likely to be mimicking the PrP^{Sc} propagation process that proceeds *in vivo*.

PMCA faithfully propagates the biochemical characteristics of the seed. The protease resistant products of PMCA resemble those of the initial PrP^{Sc} source in terms of migration in western blotting and respective ratios of glycosylated variants of PrP^{Sc} (Soto et al., 2005). This faithful propagation has been observed for experimental mouse and hamster scrapie strains, natural strains of sheep and goat scrapie, cervid and rodent CWD, ovine and bovine BSE, and human sCJD and vCJD (Jones et al., 2007; Kurt et al., 2007; Saborio et al., 2001; Soto et al., 2005; Thorne and Terry, 2008). In depth probing of the biochemical characteristics of PMCA products showed identical protease resistance profiles, relative abundance of glycosylation states, insolubility in non-ionic detergents, sensitivity to chaotropic agents, similar secondary structures as analysed by FTIR and circular dichroism, resistance to thermal inactivation and replication efficiency in PMCA as a seed in brain derived PrP^{Sc} (Castilla et al., 2005).

One of the defining characteristics of TSEs is the presence of PrP^{Sc} and associated transmissibility of disease. It is therefore crucial that PMCA products are able to cause disease if they truly represent the TSE agent. The infectiousness of PMCA products was first observed in hamster bioassays in which 100% of the PrP^{Sc} used as inoculum was mathematically verified to be generated *de novo* by sPMCA (Castilla et al., 2005). Importantly the disease presented in the hamsters, both clinically and histologically, in an identical manner to the 263K scrapie strain used as a template to generate the PrP^{Sc} via PMCA (Castilla et al., 2005). Together, these findings indicate that PMCA is able to generate

PrP^{Sc} virtually identical to the brain derived PrP^{Sc} used as a template for the misfolding process. Despite this faithful propagation of TSE strains in PMCA, the PrP^{Sc} generated is less infectious than brain derived PrP^{Sc} (Castilla et al., 2005; Deleault et al., 2007). There are many possible explanations for this disassociation, yet the distinction is important, as the ability to cause disease is a fundamental component of PrP^{Sc}. One explanation revolves around the cloud hypothesis in which a group of distinct PrP^{Sc} conformers exist with slightly different reaction kinetics and associated infectivity (Collinge and Clarke, 2007; Li et al., 2010). The conditions within PMCA might well amplify a particular conformer that is less infective, perhaps due to being more susceptible to host clearance mechanisms which do not contribute in PMCA. Alternatively the explanation may rely on the fact that sonication generates smaller aggregates of PrP^{Sc} than those found *in vivo*. Sonication has shown to reduce the size of aggregates of brain derived PrP^{Sc} conformers, and these had reduced infectiousness compared to samples of the same brain homogenate not exposed to sonication. In support of this latter explanation, PMCA products stabilised with nitrocellulose increase in aggregation size and increase in infectivity to become equivalent to that of the brain derived PrP^{Sc} used to seed PMCA (Weber et al., 2006). It may be that larger aggregates are harder to clear by the host, thus more infective.

The application of PMCA has also been much broader in recent years where it has been used to study other protein misfolding diseases. The method has been used for the successful amplification of disease associated protein aggregates include Aβ and tau associated with Alzheimer's disease and α-synuclein of Parkinson's disease (Meyer et al., 2014; Roostaee et al., 2013; Salvadores et al., 2014).

1.6.2 Improvement of PMCA

The amplification efficiency of PMCA has been improved via the addition of digitonin. Not only does digitonin increase amplification efficiency it also decreases non-template directed misfolding and generation of *de novo* prions as an artefact of PMCA (Murayama et al., 2007). Poly anionic nucleotides can also be used to improve PMCA sensitivity (Deleault et al., 2007), though this is not code specific as artificial oligonucleotides and polyadenosine RNA also improve replication (Piro et al., 2011). Polyanions are thought to act as a non-specific scaffold to aid PrP^{Sc} formation. Furthermore, the sensitivity of PMCA can be improved by the addition of Teflon beads into the PMCA reaction (PMCAb), yet this does not affect specificity (Gonzalez-Montalban et al., 2011). The beads are thought to improve sensitivity of PMCAb by increasing fragmentation of PrP^{Sc} fibrils, and subsequently generating more nucleation sites and increasing misfolding efficiency. Alternatively the beads may distribute the energy input into the system (via sonication) in a manner favouring PrP^{Sc} formation.

1.6.3 Quaking induced conversion

Quaking induced conversion (QuIC) is an *in vitro* amplification technique also used to study prions. Quaking induced conversion is similar in mechanism to PMCA but substitutes sonication for shaking (Atarashi et al., 2008). QuIC utilises recombinant PrP^{C} as substrate for conversion, which can be converted by a wide range of PrP^{Sc} strains. Traditionally hamster recombinant PrP^{C} has been used as substrate, with high sensitivity, and was able to detect 1fg PrP^{Sc} material in just 18 hours in the CSF of scrapie infected hamsters (Atarashi et al., 2008).

Further development of QuIC included the addition of thioflavin T to reaction mixes, which binds to prion amyloid formed during QuIC and fluoresces, producing a quantitative output; termed real time-QuIC (RT-QuIC) (Wilham et al., 2010).

1.6.4 Strain typing

Strain typing of prions to identify the infectious TSE agent is of particular use when assessing the identity of an infection in a food producing animal. The disease presentation of scrapie and BSE in a small ruminant host is very similar and cannot be distinguished by symptomatic differences and while no natural cases of BSE have been described in sheep, two goats have developed field cases of BSE (Eloit et al., 2005; Foster et al., 1993; Jeffrey et al., 2006b). Scrapie has no proven zoonotic threat to humans, in contrast BSE has a high zoonotic potential therefore differentiation between the two in suspect cases is extremely important. Strain typing of BSE and scrapie is further complicated by the existence of CH1641 scrapie strain, which resembles the BSE in terms of molecular weight (Foster et al. 1988). Bioassay is the current gold standard for strain typing prion infections, but requires a large amount of time to complete. Therefore sPMCA has been used to develop a rapid strain typing tool able to differentiate between CH1641 scrapie and ovine BSE (Taema et al., 2012). In this assay ovine BSE amplified readily in two ovine substrates of either VRQ/VRQ or AHQ/AHQ genotypes (at amino acid positions 136, 154 and 171) but the PrP^{Sc} of CH1641 did not amplify, allowing efficient discrimination between the two.

RT-QuIC has demonstrated great potential to act as a strain typing tool due to the wide range of human and animal PrP^{Sc} types that can seed reactions when using recombinant bank vole PrP as substrate (Orrú et al., 2015). Using this method 29 different TSE have been characterised, which include TSEs of human, cattle, ovine and rodent hosts and represent natural and experimental infections. In relation to food producing animals, isolates amplified include classical and atypical scrapie, classical, H-type and L-type BSE and CWD (Masujin et al., 2016; Christina D Orrú et al., 2015; Christina D. Orrú et al.,

2015). Recombinant bank vole substrate has been used to develop a RT-QuIC based assay for the discrimination between classical, H-type and L-type BSE (Masujin et al., 2016; Christina D Orrú et al., 2015). The assay is based on the relative seeding potential of the three BSE types in recombinant bank vole and recombinant ovine ARR (at amino acid positions 136, 154 and 171) substrates. Classical BSE will efficiently seed reactions in bank vole substrate, but cannot replicate in ovine substrate. The atypical BSEs are differentiated by comparing the seeding efficiency of ovine substrate compared to bank vole; H-type seeds ovine substrate more efficiently than bank vole substrate, for L-type the converse is true.

1.6.5 Diagnostic assays

Diagnosis of TSE diseases is problematic in both humans and animals as definitive identification requires post-mortem brain histopathology and detection of PrP^{Sc} presence. PrP^{Sc} is the only biological marker for TSE disease and progression, making its *in vitro* amplification a uniquely fitting diagnostic assay. The potential of PMCA to diagnose TSEs was first demonstrated when the assay detected PrP^{Sc} in hamster brain homogenates two weeks after inoculation with 263K scrapie (Soto et al., 2005). The same study also detected PrP^{Sc} in a cow that had been orally infected with BSE yet was free of disease symptoms and negative for PrP^{Sc} via conventional testing at the time of sacrifice. Although this technique successfully detected pre-clinical scrapie and BSE infection it relied on brain homogenates of infected animals being subject to PMCA, making it unsuitable for live animal testing.

For PMCA to be fully utilised as a pre-clinical diagnostic tool it must be less invasive and be able to utilise PrP^{Sc} seed from peripheral tissues or bodily fluids rather than brain material. To this end, scrapie has been detected in the blood of experimentally infected hamsters in pre-clinical stages of infection using PMCA, as early as 20 days post inoculation (Murayama et al., 2007; Saá et al., 2006b). PMCA has detected scrapie in the blood of naturally infected sheep, and variant CJD in the blood of infected deer (Jones et al., 2009; Thorne and Terry, 2008). Furthermore, these previous techniques have relied on the fractionation of blood prior to analysis but the seeding activity of scrapie in whole blood samples has been documented offering a highly robust and high throughput diagnostic assay (Tattum et al., 2010). Pre-symptomatic detection of PrP^{Sc} in blood offers valuable information, it could be applied to human medicine to allow early diagnosis and help prevent iatrogenic infections, it could also be used to help keep livestock free of TSEs by carrying out routine surveillance of wild and farmed animals.

Urine is another possible source of PrP^{Sc} for sPMCA analysis and has been shown to be indicative of stage and severity of disease progression. PrP^{Sc} has been detected in the urine of hamsters a few days after oral infection with scrapie, at which point PrP^{Sc} was not detected by conventional assays

(Murayama et al., 2007). Since then, CWD seeding activity has been detected in the urine of experimentally infected deer, again at a stage of disease were animals were not diagnosed as being infected by conventional tests (Haley et al., 2009b). PMCA has also been used to detect and estimate the amount of vCJD PrP^{Sc} in the urine of infected patients (Moda et al., 2014).

Another important application of sPMCA for pre-clinical diagnosis of TSEs is the detection of PrP^{Sc} in the cerebrospinal fluid (CSF) (Atarashi et al., 2007), which has been used to detect scrapie, BSE and CWD PrP^{Sc} in a range of species (Haley et al., 2013; Murayama et al., 2014; Nichols et al., 2012).

RT-QuIC has also demonstrated great potential for use as an effective diagnostic tool, as it is able to identify and differentiate acquired and inherited human TSEs: familial CJD, vCJD, sCJD, iCJD, GSS, genetic and inherited FFI (McGuire et al. 2012; Sano et al. 2013; Atarashi et al. 2011a;Orrú et al. 2015; Peden et al. 2012). In addition to the wide range of TSEs suitable for amplification, RT-QuIC has detected seeding activity from human CSF and nasal brushings of patients with sCJD, which can be used to generated ante-mortem detection assays (McGuire et al., 2012; Orru et al., 2014). Four studies using RT-QuIC to diagnose sCJD from the CSF of patients have been reported with excellent sensitivities (95.8%, 91%, 98% and 76.5%) and specificities (100%, 98%, 91% and 100%). Furthermore RT-QuIC has potential as an effective surveillance tool as a means to reduce iatrogenic transmission, able to screen blood plasma for vCJD seeding activity detecting just 2 ag of brain material (Orrú et al., 2011).

1.6.6 Defining the *in vivo* dissemination of PrP^{Sc} in food producing animals.

The *in vivo* localisation of PrP^{Sc} is particularly important in animals which are consumed by humans, to prevent TSE agents entering the human food chain. The greater sensitivity of PMCA has enabled the detection of PrP^{Sc} in a variety of tissues and animal products, including in the milk of pre-clinical scrapie infected sheep (Maddison et al. 2009; Gough et al. 2009). The method has also been used to identify BSE in peripheral lymph ((Franz et al., 2012; Gough et al., 2009; Maddison et al., 2009). CWD is present in both the wild and farmed deer and elk populations in North America, both of which are consumed by humans. CWD prions have been shown to be present with the nerve bundles of muscle fibres, antler velvet, salivary gland, intestinal tract, bladder, tongue and kidney, amongst other tissues including brain and blood, using PMCA (Angers et al., 2009; Daus et al., 2011; Haley et al., 2011). Using PMCA to determine tissues with relatively high PrP^{Sc} levels in food producing animals is extremely important to inform risk assessments to minimise the entry of TSE agent into the human food chain.

The wide range of tissues and fluids that have been used to detect seeding activity in RT-QuIC, particular with respect to CWD of deer and elk has made it a test with significant diagnostic potential

Direct application of QuIC to CSF has and it has also been shown to detect seeding activity of prions from sheep scrapie and deer CWD (Haley et al., 2013; Orrú et al., 2009). RT-QuIC has assessed dissemination routes of scrapie after different challenge locations in hamsters (Orrù et al., 2012) and CWD in the peripheral cervid lymph nodes in natural infections (Haley et al., 2014). RT-QuIC has also been applied to the detection of scrapie and CWD PrP^{Sc} in the whole blood of hamster of deer (Elder et al., 2015). Furthermore CWD has been detected from nasal brushings and rectal mucosa lymphoid tissues (Haley et al., 2016) In addition to this RT-QuIC has detected seeding activity in the saliva, urine and faeces of CWD infected deer including in asymptomatic cases, highlighting this assay as a promising candidate to use as in non-invasive surveillance of wild deer populations (Henderson et al., 2015b, 2013; John et al., 2013). The seeding ability of TME has been found in olfactory sensory epithelium in infected hamsters (John et al., 2013).

1.6.7 Environmental TSE Detection

The apparent abundance of PrP^{Sc} in secretary organs, saliva, blood, milk, semen, urine and faeces of a range of animals infected with different TSEs implies that prion may be excreted from the animals into the environment, for both farmed and wild animals, and act as a reservoir for infection (Balkema-Buschmann et al., 2011; Haley et al., 2011, 2009b, Maddison et al., 2010c, 2009; Murayama et al., 2010; Rubenstein et al., 2012; Terry et al., 2011). To this end PMCA has been used to find prion seeding activity in a wide range of environmental sources. Prions are extremely stable and can persist in the soil and water run off for up to 29 months retaining both PMCA seeding activity and infectivity in bioassay (Seidel et al., 2007). Furthermore scrapie on an Icelandic farm has been demonstrated to persist for at least 16 years in the environment before infecting sheep resulting in a scrapie outbreak (Georgsson et al., 2006). The level of seeding activity retained in soil appears to be dependent on the source of TSE, soil type and temperature (Maddison et al., 2012, 2010b; Saunders et al., 2011). Importantly there is some evidence that strain interaction, conversion or evolution can occur during prolonged soil association (Maddison et al., 2015). Natural environmental water samples have also been shown to contain enough CWD PrP^{Sc} to seed PMCA reactions in areas with endemic infections (Nichols et al., 2009). On a scrapie endemic farm, PrP^{Sc} has been detected on the surfaces of a wide range of indoor and outdoor objects that come into contact with infected sheep. These infected areas include pens, gates, water and feed troughs, fence posts made up of different materials: plastics, wood and metal (Maddison et al., 2010a).

1.6.8 Assessing decontamination procedures

The stability of PrP^{Sc} makes routine de-contamination procedures for exposed environments or materials difficult. PMCA has been utilised to assess various heating and chemical treatment as means to reduce prion load (Murayama et al., 2006; Russo et al., 2009; Suyama et al., 2007). PMCA has shown the effectiveness of autoclave inactivation of PrP^{Sc} and with sensitivity comparable to bioassay (Matsuura et al., 2013; Murayama et al., 2006; Yoshioka et al., 2013). Decontamination of prion contaminated environments can be problematic, as it is unsuited to combinations of harsh heat and chemical treatments. As such PMCA has been used to assess novel chemical treatments more suitable for environmental use (Saunders et al., 2011; Suyama et al., 2007).

1.6.9 Quantitative applications of *in vitro* amplification methods

One of the major drawbacks of the *in vitro* amplification processes is the lack of quantitative data that can be generated, in contrast bioassays produce LD_{50} values for reliable quantitation of TSE infectivity. Adding a quantitative output to PMCA enables *in vitro* amplification techniques to estimate the relative seeding activity (and in some cases this can be associated with prion infectivity and PrP^{Sc} levels) of a sample. Quantitative PMCA (qPMCA) can test different disinfection and decontamination techniques as well as estimate prion levels in different tissues.

A quantitative output is assigned to PMCA by comparing the replication efficiencies of a reference sample to the unknown test samples. The reference sample is usually a TSE isolate that has already been measured for infectivity via bioassay, has a defined replication efficiency in sPMCA or that has had its PrP^{Sc} levels quantified via comparison to recombinant PrP stock of known concentration (Chen et al., 2010; Pritzkow et al., 2011; Saunders et al., 2011).

The replication efficiency of both the reference and unknown sample can be defined as the serial dilution at which 50% of the replicates produced positive PMCA signals (SD_{50}) which is analogous to LD_{50} values produced in bioassays. It has been suggested that there is a disassociation between infective particles and those capable of seeding PMCA reactions (Castilla et al., 2005; Deleault et al., 2007). Routinely the LD_{50} values are higher than SD_{50} values indicating they have a greater sensitivity and can detect seeding particles that do not cause diseases in bioassay (Makarava et al., 2012). LD_{50} values could also be much larger than SD_{50} counterparts because the PMCA is only an *in vitro* model for actual animal infections, as such they are over simplified and key factors that contribute to LD_{50} values do not affect SD_{50} values. One factor is host clearance mechanisms that actively remove aggregates of PrP^{Sc}, which is not present in PMCA.

In the first published qPMCA study, 263K PrP^{Sc} was precipitated from infected hamster brain homogenates, and the concentration of PrP^{Sc} was estimated in the precipitate by comparing the ELISA signals to a recombinant PrP stock of known concentration (Chen et al., 2010). This 263K scrapie became the reference sample and by comparing the SD_{50} of the reference and those of tissues from 263K infected hamsters the authors were able to describe the relative levels of prions throughout those tissues. Of the tissues analysed, brain contained the highest concentrations of PrP^{Sc} followed by spleen, blood then urine. The results indicated that in brain there is $2.3x10^{-5}$ g PrP^{Sc} /g tissue, which is $1x10^{6}$ fold more than in the spleen and $1x10^{11}$ fold more than found in the urine of these hamsters.

qPMCA has also been utilised to assess the effectiveness of environmental and medical decontamination techniques. Environmental contamination with prions poses a difficult challenge, as decontamination methods have usually been developed for laboratory or medical instruments and equipment and are usually unsuitable in an environmental setting. Once a disinfection technique has been devised it must then be tested against current practises for reducing infectivity of the contaminant. To this end qPMCA has been used to assess the ability of bacterially expressed subtilisin to reduce the infectivity of hyper TME in different soil types (Saunders et al., 2011).

There are documented cases of iatrogenic CJD resulting from surgery and blood transfusions (Jones et al., 2009; Wadsworth et al., 2001). PrP^{Sc} is stabilised by stainless steel (Zobeley et al., 1999) and therefore the source of some of these infections could be contaminated instruments and there is a need for effective decontamination procedures. qPMCA has been used to assess a range of chemical decontamination techniques, using 263K scrapie as a model TSE which was bound to steel wires (Pritzkow et al., 2011). The initial contamination of steel wires and subsequent reduction in infectivity after sterilisation treatment was quantified using qPMCA, and further verified in bioassay with good correlation between relative $PMCA_{50}$ and LD_{50} values. Different TSEs show different biochemical properties and susceptibilities to chemical decontamination regimes, therefore vCJD was later analysed in an analogous way using qPMCA to determine the effectiveness of decontamination procedures (Boerner et al., 2013).

Additionally, qPMCA has been proposed in medical research as a surveillance and diagnostic tool for human prion diseases. vCJD prions have been successfully identified in the urine of vCJD positive patients but crucially not in patients with other protein misfolding diseases or sporadic or familial CJD (Moda et al., 2014). qPMCA was able to estimate levels of vCJD prions in these infected individuals with 93% sensitivity and 100% specificity, and may be used to inform disease epidemiology and disease progression in the future.

The titre and localisation of infectivity is also crucially important in farmed animals for ensuring good animal husbandry, informing best practises and the treatment of specified risk materials. Practises

used by the livestock industry have come under scrutiny since the BSE epidemic and the need to reduce infectivity of known contaminated animals. To this end, qPMCA has been employed to measure how well wet heat (Matsuura et al., 2013) and treatment in yellow grease (Yoshioka et al., 2013) reduces infective titre. The ability of heat treatment in yellow grease to reduce infective titre is particularly important as it is used to produce meat and bone meal feed. Both studies found that increasing temperature and heating time reduced infectivity, but only to a cut-off temperature. They theorise at this temperature charring of the carcasses by excessive heat reduces thermal penetration and effective titre reduction (Matsuura et al., 2013; Yoshioka et al., 2013).

Quantitative output has also been applied to RT-QuIC assays to estimate the PrP^{Sc} content for a range of applications. Quantitative RT-QuIC is achieved in a similar manner to qPMCA, a serial dilution of prion infected seed is amplified using RT-QuIC, to find the dilution at which on 50% of the replicates are positive (SD_{50}) (Wilham et al., 2010). This technique was first introduced to estimate the prion load in nasal lavage of hamster infected with TME, and in the CSF of scrapie infected hamsters, and found high seeding activity in the nasal lavage. However definitive correlation of SD_{50} and LD_{50} was not established (Wilham et al., 2010). RT-QuIC has been used to quantify the tissue distribution of scrapie infected mice and CWD infected deer in time point infection studies to assess the PrP^{Sc} dissemination during disease progression (Shi et al. 2013; Henderson et al. 2015). Mice infected with scrapie revealed seeding activity in the brain, spleen and muscles of infected mice, which increased with time post inoculation (Shi et al., 2013). CWD infected deer during infection developed seeding activity in several tissues, including brain, pancreas spleen and even in saliva and urine (Henderson et al. 2015).

1.7 Bioassays for prion diseases

After the initial description of scrapie, study of the disease progressed through natural field cases and exposure of healthy sheep to infected individuals and contaminated environments (Greig, 1950). In 1935 the accidental exposure of around 18,000 sheep to a scrapie contaminated louping-ill vaccine and the subsequent 1500 confirmed scrapie cases proved in principal that the infection could be propagated from animal to animal artificially (Brown et al., 1982; Greig, 1950). Demonstrations of experimental scrapie transmissions were published in 1936 by Cuile and Chelle in which intraocular, intrathecal, intracerebral and subcutaneous injections of scrapie contaminated CNS tissues caused disease in healthy sheep (Greig, 1950). The scrapie agent was demonstrated to be transmissible to goats in this same manner, and then later to a range of rodents, including mice, rats and hamsters (Chandler, 1961; Chandler and Fisher, 1963; Zlotnik and Rennie, 1965). These studies formed the basis of the animal bioassays which typically used rodents intracerebrally inoculated with prions to assess

the infectious titre and strain type of the prions (Prusiner, 1998). Infectious titres can be calculated by producing an end-point dilution series of a prion contaminated sample which is used to infect a test group of animals (Prusiner et al., 1981a).

Much information can be gained from animal bioassays that can be used to strain type prions which typical occur in mice. Following serial passage of prions the incubation time before onset of clinical disease and the attack rate of disease in the subjects is reproducible and specific to strains of scrapie and BSE (Bruce et al., 1991). The pattern and severity of spongiosis in the brain is also specific for prion strains due to the preference each strain exhibits for replication in particular brain regions (Bruce et al., 1991; Fraser and Dickinson, 1967). Further immunohistochemical techniques can be applied to determine the pattern of PrP^{Sc} deposition in the brain, the peripheral tissue distribution of PrP^{Sc} and the antibody reactivity of PrP^{Sc}, all of which provide extra detail in determining prion strain (Bruce et al., 1989; González et al., 2003; Jeffrey et al., 2006b; Jeffrey and González, 2007).

The advent of transgenic DNA technologies further improved the efficiency of these assays, enabling rodents to express ovine, bovine and human genotype PrP, which effectively ablated transmission barriers to prion infections thus reducing the incubation periods significantly. Transgenic mice have been instrumental for conducting fundamental research on TSEs (Prusiner et al., 1990). Atypical H-type and L-type BSE fail to transmit to wild type mice, yet they can replicate faithfully in mice expressing bovine PrP, along with classical BSE, enabling differentiation in these mice (Masujin et al., 2008; Okada et al., 2011).

While bioassays are able to produce reliable data in relation to infectious titre, strain types and fundamental prion biology they do have limitations. Animal bioassays can take years to conduct and require large numbers of individuals to be infected. This means animal bioassays are expensive assays to conduct with large ethical implications, and the time taken to perform just one round of animal bioassay is not conducive to a modern day research environment.

Aims of the study

The aims of this study were to apply the *in vitro* amplification technique, sPMCA, to prions which infect ruminants to aid both detection and characterisation of these diseases.

1. **Develop an *in vitro* amplification method based on recombinant hamster PrP as substrate to measure the infectivity of scrapie.**

   The current gold standard for measuring the infective titre of prion is rodent bioassay. While bioassays can produce accurate measures of prion infectivity, application of this assay is limited due to the high costs and large amount of time that is required to complete the assay. Quantifying prion content of samples has many applications, it can be used to study tissue distribution of PrP^{Sc} and coupled with a time point infection study this may enable better understanding of prion disease progression and identify high risk animal products. Furthermore understanding prion load in different environmental conditions could lead to better farming practices to reduce prion outbreaks and assess decontamination techniques. *In vitro* amplification of prions by sPMCA offer a potential alternative to bioassays, as they are considerably faster and cheaper and there is some evidence for correlation between the *in vitro* amplification efficiency in sPMCA and the infectivity in bioassay of PrP^{Sc} (Yoshioka et al. 2013; Matsuura et al. 2013; Samuel et al. 2011). To study this we sought to (i) develop a recombinant hamster PrP based amplification technique (ii) use this technique to assess correlation between amplification efficiency and infectivity.

2. **Application of *in vitro* amplification to H-type and L-type BSE for detection and discrimination from classical BSE.**

   Amplification of atypical BSE isolates using *in vitro* amplification techniques has been limited. H-type BSE has not previously been amplified in sPMCA, while L-type amplification has only succeeded using a cocktail of additives which included L-arginine ethylester and polyanions (Murayama et al., 2016). Both L-type and H-type BSE have been amplified using RT-QuIC, but discrimination between the two can only be accomplished at 10^{-3} and 10^{-5} dilutions of seed material (Orrú et al. 2015; Orrú et al. 2015b; Masujin et al. 2016). Atypical BSE infections which are mainly asymptomatic, occur in older animals compared to classical BSE and in infection models atypical BSE appears to pose a larger than classical BSE (Béringue et al., 2008; Brown et al., 2006; Comoy et al., 2008; Kong et al., 2008) Therefore there is need for a sensitive *in vitro* assay capable of detecting and discriminating minute quantities of atypical BSE PrP^{Sc} to study atypical BSEs and assess the incidence of this disease in older cattle. To accomplish this we set about developing sPMCA methods capable of amplifying atypical BSE and

characterising the amplified PrP^{Sc} by protease sensitivity, antibody reactivity and conformational stability.

3. **Further characterise the sensitivity and specificity of an sPMCA assay capable of preferential amplification of BSE PrP^{Sc} in comparison to current statutory methods.**

   A modification of sPMCA has recently been described in which ovine substrates are used to selectively amplify BSE PrP^{Sc} despite the presence of excess scrapie in the seed (Gough et al., 2014). The assay alternates the genotype of brain homogenate used as substrate for amplification between AHQ/AHQ and VRQ/VRQ (at amino acid positions 134, 156 and 171) and achieved sensitivity of 97% and specificity of 100% when seeds contained 1% BSE and 99% scrapie (v/v). The aim of this study was to compare this assay to mouse bioassays, western blotting and ELISA in terms of sensitive and specific detection of BSE PrP^{Sc} when present with excess scrapie. Therefore samples containing various concentrations of BSE and scrapie infected 10% (w/v) brain homogenates were amplified in the sPMCA assay and tested in current statutory tests by collaborators in a blind trial.

4. **Assess to what extent does co-infection with scrapie effect BSE PrP^{Sc} tissue distribution in an ovine host.**

   Very limited research on TSE co-infections has been carried out, and none addressing the prospect of scrapie and BSE co-infection in an ovine host. We set out to assess if BSE and scrapie co-infections can occur, and if so what effect did this have on the tissue distribution of BSE. To address these questions we applied the alternating substrate sPMCA assay, which has demonstrated excellent sensitivity and specificity for BSE when applied to mixtures containing both scrapie and BSE, to the brain, spleen and lymph of sheep experimentally infected with BSE and scrapie (Gough et al., 2014; Simmons et al., 2015). Furthermore we sought to compare how the sensitivity and specificity of the sPMCA compared to current strain typing assays, consisting of western blotting, neuropathology, immunohistochemistry, and mouse bioassay, which were conducted by collaborators.

5. **Characterise the scrapie like molecular profile of BSE PrP^{Sc} as a result of *in vitro* amplification in ovine substrate.**

   During the course of this PhD, amplification of BSE in sPMCA using ovine VRQ/VRQ as substrate demonstrated a molecular switch in the PrP^{Sc} to a high molecular weight and antibody reactivity profile of scrapie. We sought to reproduce this phenomenon and thus

demonstrate the molecular switch was reproducible in an independent laboratory using different BSE isolates. Furthermore we aimed to compare the PrP^{Sc} of the scrapie like PrP^{Sc} to that of genuine BSE and scrapie PrP^{Sc} in terms of molecular weight, antibody reactivity, glycoform profile and conformational stability.

Chapter 2. Materials and methods

2.1. Handling of TSE materials.

All materials potentially contaminated with TSEs were handled in compliance with local and national regulations, which include the HSE approved guidelines. All work involving TSE materials were carried out in a TSE specific containment laboratory (category 3 with derogations) and any potentially infectious materials were handled within either a class I/III hybrid or class II microbial safety cabinets (Contained air solutions, UK). Decontamination and waste disposal regimes were in accordance with the local guidelines.

2.2. Sourcing of healthy and infectious animal material

TSE infected and healthy animal materials were sourced from the Roslin institute and the APHA biological archive. For details of healthy (table 1), scrapie infected (table 2) and BSE infected (table 3) material see appendix.

2.3. Preparation of 10% brain homogenates

Three different methods were used to produce brain homogenates that were included in this thesis. Method 1 was used to process all uninfected 10% brain homogenates from specific TSE free flocks and herds for use of substrate for *in vitro* conversion experiments. The majority of infectious brain homogenates were created using method 2. Method 3 was specifically used to process homogenates of brain, spleen and prescapular lymph node samples provided by the Roslin institute (Chapter 6).

2.3.1. Method 1

Uninfected brain homogenates were prepared outside of the containment laboratory. The outer membranous tissue and exterior blood vessels were removed and the brain flash frozen in liquid nitrogen then stored at -20^{o}C for use at a later date. The brain was then weighed and processed for 20 seconds in a BL09 blender (Tesco, UK) with an appropriate amount volume of conversion buffer to produce 10% homogenates (w/v). Conversion buffer consisted of phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with the addition of 150 mM NaCl, 1% (v/v) Triton X-100, 4 mM ethylenediaminetetraacetic acid (EDTA) and complete protease inhibitor (Sigma-Aldrich, UK). The homogenate was further processed using a HBB908 bead beater (Thistle Scientific, UK) and glass beads (VWR, UK) for 30 seconds. The homogenate was then decanted into storage tubes and stored and -80^{o}C.

2.3.2. Method 2

Brain containing infectious TSE material was cut into small pieces using a scalpel blade and diluted with brain homogenate lysis buffer to give a 10% homogenate (w/v). This homogenate was then passed through progressively smaller gauge needles (19G, 21G and 23G) to produce a fine homogenate. The debris was discarded after centrifugation at 2000 rpm in a benchtop hereues megafuge 11R (Thermo Fisher Scientific, USA) for 5 minutes and the supernatant retained and stored at -80^{o}C.

2.3.3. Method 3

Brain, spleen and lymph nodes used in chapter 6 were homogenised by weighing a small amount of tissue and placing it into reinforced 2 ml tube (Sarstedt, Germany) containing enough glass beads to reach the 1 ml marker (Stratech, UK) and relative volume of brain homogenate lysis buffer for a 10% homogenate (w/v). Sealed tubes were shaken in a 3110BXEUR mini-bead beater (Stratech, UK) at 4800 rpm for 60 seconds for brain and spleen and 180 seconds for prescapular lymph nodes. Homogenates were then transferred to a fresh 1.5 ml screwcap tube using a blunt needle and syringe and stored at -80^{o}C.

2.4. Serial protein misfolding cyclic amplification

Uninfected 10% brain homogenates, of various genotypes at PrP residues 136, 154 and 171, were used as substrate and aliquoted into 0.2 ml PCR tubes (Sarstedt, Germany). Either 10 µl or 1 µl of TSE infected brain homogenate (spike) was added making the final volume of 100 µl per PMCA reaction. Sealed tubes were incubated at 37^{o}C in a Misonix S-4000 ultra-sonicating water bath (Misonix, USA) for 48 cycles comprised of incubation for 29 minutes 20 seconds incubation followed by 40 seconds of sonication (unless otherwise stated). The power was set to 50% maximum output (~200W). 48 cycles of incubation and sonication constituted one round of PMCA, after each round of PMCA the products of amplification were diluted 1 in 3 into fresh substrate (total volume 100 µl) and incubated again in the ultra-sonication water bath for another 48 cycles. Typically serial PMCA reactions were carried out for 5 rounds. Products of amplification were stored at -80^{o}C.

2.5. Serial protein misfolding cyclic amplification to discriminate between BSE and scrapie

To differentiate between BSE and scrapie isolates a previously described PMCA technique was used (Gough et al., 2014). The PMCA procedure was similar to that detailed above but with minor modifications: Spikes of 10 µl were amplified using the PMCA conditions described previously with the first round of amplification using substrate of genotype AHQ/AHQ at amino acid positions 136, 154, and 171 respectively. The products were diluted 1 in 3 in round 2, using substrate of VRQ/VRQ genotype, and each subsequent round the genotype is alternated between AHQ/AHQ and VRQ/VRQ genotype substrates. Five rounds of amplification were performed.

2.6. Alternative method of serial protein misfolding cyclic amplification

An alternative PMCA method was used in chapters 7 for the amplification of bovine BSE which has previously been published (Krejciova et al., 2014b). TSE positive spike was diluted 1 in 10 in VRQ/VRQ or AHQ/AHQ substrate, with a final volume of 100 µl, 0.2 ml tubes were then sealed and placed in a Misonix S-4000 ultra-sonicating water bath (Misonix, USA) for one round of PMCA. Each round of PMCA consisted of 96 cycles of 30 minutes of incubation at 37°C, in the last 20 seconds of the incubation period samples were sonicated at 200W. Following each round of amplification PMCA products were diluted 1 in 10 in fresh substrate, and the PMCA round repeated, for a total of 9 rounds of amplification.

2.7. Digestion of PrP^{C} and PrP^{Sc} using proteinase K

PMCA products and unamplified 10% (w/v) brain homogenates were detected first by digestion with proteinase K (PK) (Sigma-Aldrich, UK). Samples of 20 µl was diluted with equal volume of PK and buffer. PK and buffer were produced from stocks of PK at 50 mg/ml and diluted into PBS typically for a final concentration of 50 µg/ml and sodium dodecyl sulfate (SDS) final concentration of 0.04% (w/v). Other final concentrations of PK were used for various PK sensitive products including 25, 10 and 5 µg/ml. Samples were digested by incubation in a water bath at 37°C for 1 hour. In some instances mild and stringent digestion conditions were compared, in these cases mild reaction buffer consisted of 200 mM monopotassium phosphate (pH 6.5) and stringent buffer of 200 mM Tris base (pH 8.0), these digestions were based on those performed by Jacobs and colleagues (Jacobs et al. 2007).

2.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Digested samples were diluted in NuPAGE LDS sample buffer (Invitrogen, UK) and 5% (v/v) β-mercaptoethanol before being boiled at 100^{o}C for 10 minutes. Boiled samples were then loaded on 12% Bis-Tris NuPAGE Novex gels (Invitrogen, UK) and ran at 150 V for 90 minutes in MOPS running buffer (Invitrogen, UK) (50 mM MOPS, 50 mM Tris Base, 0.1% (w/v) SDS 1mM EDTA, pH 7.7). Protein was transferred from the gel to polyvinylidine difluoride membrane (PVDF) (Roche, UK) via western blotting at 30V for 75 minutes in transfer buffer (Invitrogen, UK). PVDF membranes were blocked overnight in trisaminomethane buffered saline with 1% (v/v) tween 20 (TBST) (140 mM NaCl, 2.5 mM KCl, 25 mM tris base) with 5% (w/v) milk powder (SMA). Membranes were then probed with SHa31 (Bertin Pharma, France) at 1 in 80,000, P4 (R-Biopharm AG, Germany) at 1 in 1000 or SAF84 (obtained from Centro de Investigacion en Sanidad Animal, Spain) at 1 in 1000 in TBST 0.5% (w/v) SMA skimmed milk powder (SMA). Primary antibodies SHa31 and SAF84 recognise core epitopes, P4 epitope is located near the N-terminal (figure 2.1). Following 2 hours incubation membranes were washed 3 times in TBST 0.5% (w/v) SMA and probed with secondary antibody, goat anti-mouse HRP conjugate (Dako, Denmark) at 1 in 40,000 in TBST 0.5% SMA for 1.5 hours. Blots were washed 4 times in TBST 0.5 % SMA then visualised using EZ-ECL chemiluminescent substrate (Biological industries, Isreal) in an ICCD225 photon counting camera (Photek, UK).

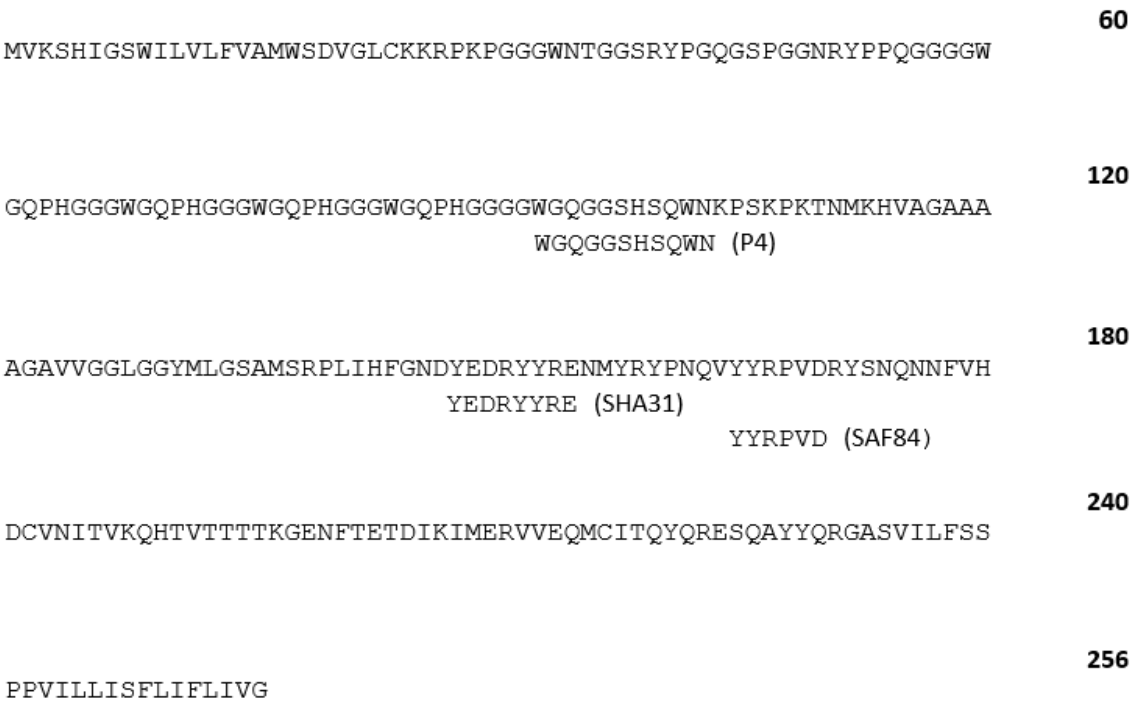


Figure 2.1. Primary antibody epitopes in ovine PrP. The Epitopes of primary antibodies P4, SHa31 and SAF84 mapped to the amino acid sequence of ovine PrP (EMBL accession number P23907).

2.9. Visualising of digested PrP^{Sc} using dot blotting

Digested samples were diluted 3 in 4 with NuPAGE LDS sample buffer containing 5% (v/v) mercaptoethanol and boiled for 10 minutes. 2.5 µl of boiled sample was carefully spotted onto a 1 cm^2 area of nitrocellulose membrane (GE Healthcare, UK) (containing an appropriate 1 cm^2 grid). The nitrocellulose membrane was allowed to dry for a minimum of 30 minutes and blocked overnight in TBST 5% (w/v) SMA. Blots were probed with 20 ml SHa31 antibody (1 in 40,000) in TBST 0.5% SMA, sealed in a plastic wallet and rotated on a vertical rotating shaker for 2 hours. Blots were washed 3 times with TBST 0.5% SMA for 10 minutes and probed with 20 ml goat anti-mouse HRP conjugate (Dako, Denmark) antibody diluted 1 in 2000 in TBST 0.5% SMA. Blots were sealed in plastic wallets and rotated for 1.5 hours then washed 4 times with TBST 0.5% SMA for 10 minutes. Dot blots were then visualised using EZ-ECL chemiluminescent substrate and a photek photon counting camera.

2.10. Densitometry of western blots and dot blots

Various densitometric analyses were undertaken on western blots and dot blots all of which used the software ImageJ (National Institutes of Health, USA). To find the signal value for any particular lane on

a western blot the rectangle tool was used to define the lanes which were then expressed graphically using the plot lanes function. The background was subtracted from the peaks on these graphs using the straight line tool to define the peaks and the signals of the peaks were then found by selecting them with the wand tool.

In Chapters 5, 6 and 7 in which alternating substrate was used as a method to determine the identity of unknown TSEs as scrapie or BSE, a general background threshold value of blots was produced. This value was three standard deviations from the mean signal of 50 lanes that contained only scrapie sPMCA products which had been digested with 50 µg/ml PK and probed with SHa31. All PMCA products analysed via densitometry had to exceed this background value to be considered a positive result. To find the identity of an unknown sample the same digest was analysed in SDS-PAGE and western blotting (as described previously) in duplicate and probed with both SHa31 and P4. If the SHa31 signal exceeded the background threshold value then a ratio of SHa31:P4 signal was produced and expressed as a function of an internal scrapie control blotting control. If this control adjusted ratio exceeded 2.9 it was considered to be a BSE profile, and anything below 2.9 a scrapie profile.

ImageJ was used to analyse the glycoform profiles of PrP^{Sc} from various 10% (w/v) brain homogenates and PMCA products. Each lane was selected manually using the rectangle lane tool. Each PrP^{Sc} glycoform peak was defined from the blot background and the signal of each peak was measured using the wand tool. The signal of the diglycosylated and monoglycosylated PrP^{Sc} were expressed as a percentage of the total signal of all three glycotypes. The percentage values were plotted against each other in an XY scattergraph using prism software (graphpad, USA).

To determine the molecular weight of various glycosylated species of PrP^{Sc} in PMCA products and brain homogenates the relevant western blot images were loaded into image J. First the molecular markers were selected using the rectangle tool then the lanes were selected using the rectangle tools. The selections were analysed using the plot lane function and each peak of the generated graphs labelled using the multi-point tool. The relative migration values for each peak (x values produced by image J) for the molecular markers was plotted against the known weight for each protein in the marker. A linear regression analysis was used to produce a standard curve from the molecular markers and the molecular weights of the unknown peaks were estimated by interpolating from the standard curve.

Output of dot blots was processed using the “dot blot analyzer for ImageJ” plug in (available at http://image.bio.methods.free.fr/dotblot.html). Using the single measurement tool, with radius set to 16 (arbitrary units), the signal of every dot was measured and generated into a table that was saved as a Microsoft excel file. Each qPMCA blot analysed by densitometry contained 10 replicates of digested substrate. The signals of these negative control dots were used to generate a background

signal cut off threshold for the dot blots. The threshold was three standard deviations from the mean of the substrate only negative controls. Test samples that exceeded this value were considered to be positive.

2.11. Conformational stability assay

Conformation stability assays were applied to a range of BSE and scrapie 20%, 10% and 1% (w/v) brain homogenates and sPMCA products. The method was based on previously described by Peretz and colleagues (Peretz et al., 2001), 50 µl of 10% (w/v) brain homogenate or amplified sPMCA products were buffered with an equal volume of 100 mM Tris-HCl and incubated at 37^{o}C for 1 hour with intermittent gentle agitation. This sample was split into 9 individual 10 µl aliquots which were mixed with equal volume of guanidine hydrochloride (Gdn-HCl) (Sigma-Aldrich, UK) to produce a dilution series with final concentrations between 0.0 M and 4.0 M Gdn-HCl at 0.5 M increments. The dilution series was incubated for 1 hour at 37^{o}C with gentle agitation. The Gdn-HCl dilution series were then all diluted to 0.4 M Gdn-HCl with a final volume of 400 µl and digested with 50 µg/ml PK (H-type BSE PMCA products and relevant controls were digested at 10 µg/ml) for one hour at 37^{o}C. The activity of PK was stopped with 5 mM PMSF (VWR, UK) and proteins precipitated in 1 ml methanol overnight at -20^{o}C. The protein was pelleted at 17,000 x g in a microfuge and the supernatant discarded. Dried pellets were resuspended in 15 µl of 2 times NuPAGE LDS sample buffer (Invitrogen, UK) and 5% (v/v) β-mercaptoethanol and boiled for 10 minutes at 100^{o}C. Boiled samples were loaded onto 12 % Bis-Tris NuPAGE Novex gels (Invitrogen, UK) transferred to PVDF membrane (Roche, UK) and probed with antibodies as described previously. Using ImageJ software the signal of each point of the Gdn-HCl dilution series was measured, and expressed as a percentage of the 0.5 M signal value and plotted as an XY scatter graph using GraphPad Prism software. In GraphPad Prism, sigmoidal dose-response curves were fitted to the graphs, and Gdn_{50} values generated by interpolating values at 50% denaturation by Gdn-HCl (relative Gdn_{50}). The percentage of signal at 3.0 M Gdn-HCl was interpolated from the sigmoidal dose-response curves as another measure of resistance to Gdn-HCl. The variance of Gdn_{50} and signal at 3.0 M Gdn-HCl was analysed in a one way ANOVA using the statistical software package built into GraphPad Prism, multiple comparisons were analysed using Tukeys statistical hypothesis.

2.12. Growth and expression of recombinant protein in *Escherichia coli*

E. coli (DE3 strain) containing expression vector (pET22b) which encoded hamster prion protein was streaked onto 2 times yeast-tryptone agar plates (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 15g/l

bacteriological agar) with 100 µg/ml ampicillin and grown overnight at 37°C. A single colony of bacteria was picked and grown overnight in 10 ml two time yeast-tryptone media (2YT)(16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) containing 100 µg/ml ampicillin and 3% (w/v) glucose (2YT-AG) at 37°C in a shaking incubator, 200 rpm. Following overnight growth the culture was diluted 1 in 100 into fresh 2YT-AG media and incubated at 37°C and 200 rpm shaking until OD_{400} of 0.4 was achieved. Bacterial cells were pelleted at 3000 rpm in hereues megafuge 11R (Thermo Fisher Scientific, USA) for 30 minutes. The supernatant was discarded and bacteria resuspended in 250 ml 2YT media with 100 µg/ml ampicillin and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown overnight at 37°C with agitation at 200 rpm. Cells were pelleted at 3000 rpm in hereues megafuge 11R, the supernatant discarded and pellets stored at -20°C overnight. Frozen pellets were resuspended in 10 ml lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10 mg/ml lysozyme, 0.1% (v/v) Nonidet P-40 (VWR, UK) and complete protease inhibitor (Roche, UK) and incubated at 37°C for 1 hour. 240 µl of 1 mg/ml DNase I (New England Biolabs, UK) and $MgCl_2$ at 0.1 mM were added to the lysing cells and incubated at room temperature for 15 minutes. Samples were then spun at 23,000 g for 20 minutes, the supernatant was discarded and pellets resuspended in 25 ml wash buffer (10 mM Tris, 0.01% (v/v) Nonidet P-40) and incubated on ice for 20 minutes. Pellets were washed in this manner a further 3 times. The lysed cells were centrifuged once more and resuspended in 2.5 ml solubilisation buffer (8 M urea, 50 mM NaH_2PO_4, 300mM NaCl, pH 7.5), to denature proteins and prevent the aggregation of proteins, and the sample was then rotated overnight. Cell debris was pelleted at 23,000 g and supernatant removed and stored at 4°C.

2.13. Fast protein liquid chromatography (FPLC) purification of recombinant PrP

A Hi-trap sepharose chelating column (GE healthcare, UK) was charged with 50 mM copper sulphate to immobilise copper ions for immobilised metal affinity chromatography, on an AKTA prime FPLC machine (GE healthcare, UK). The column was primed with solubilisation buffer and then denatured proteins loaded onto the column at 0.2 ml/min. The octapeptide repeat within the prion protein binds to divalent cations, including copper, directly (figure 1.1). The column was washed using a gradient wash function (from 8 M to 0 M urea) to both remove any nonspecific column binders and to refold bound recombinant PrP into its native state. Bound proteins were eluted into fractions using imidazole in elution buffer (50 mM NaH_2PO_4, 300mM NaCl, 0.5 M imidazole pH 7.5), which has a higher affinity to the immobilised copper ions than recombinant PrP and as such displaces it from the column. These fractions were diluted into NuPAGE LDS sample buffer and analysed by SDS-PAGE. The gels were removed from the cassettes and stained with 25 ml 0.5% coomassie blue R-250 (VWR, UK) for 2 hours and destained overnight with acetic acid solution. Fractions containing recombinant

PrP were pooled and the concentration estimated using the Bradford assay. Concentrations of bovine serum albumin (BSA) (Sigma-Aldrich, UK) at 0.00, 0.25, 0.50, 1.00 and 1.40 mg/ml were used to produce a standard curve. 50 µl of pooled PrP containing fractions and BSA calibration standards were mixed with 1.5 ml Bradford reagent (Fisher Scientific, UK) and the optical density read at 595 nm. The standards were used to generate a linear regression curve from which the concentration of the PrP samples could be estimated. To ensure minimal protein degradation, pooled PrP fractions were stored in 20% (w/v) sucrose at -20°C.

2.14. Serial protein misfolding cyclic amplification with recombinant substrate

10 µl of TSE spike (or 10% (w/v) TSE-free brain homogenate no spike control) was amplified in 75 µl of substrate. Recombinant substrate contained conversion buffer made up of PBS 0.05% (w/v) SDS and 0.05% (v/v) Triton X-100 6% (w/v) sucrose and 0.1 mg/ml recombinant hamster prion protein. Sealed PCR tubes were incubated at 37°C in an ultra-sonicating water bath for 30 minutes, the final 40 seconds of which the tubes were sonicated at 50% total power output (200W). This incubation and sonication pattern was cycled 48 times which constituted one round of PMCA. Following a round of amplification, PMCA products were diluted 1 in 3 into fresh recombinant substrate. Recombinant PMCA lasted for 2 or 3 rounds, at which point the final PMCA products were digested with PK and analysed using SDS-PAGE and western blotting.

2.15. Precipitation of PrP^{Sc} using sodium phosphotungstic acid (NaPTA)

NaPTA (Sigma-Aldrich, UK) was added to PMCA products or 10% (w/v) brain homogenates, previously digested with 50 µg/ml PK, to a final concentration of 0.3% (w/v) NaPTA and incubated for 30 minutes at 37°C. This caused proteins to precipitate which were pelleted in a Heraeus Pico 17 microcentrifuge (Fisher Scientific, UK) at 17,000 g and the supernatant discarded. Pellets were then suspended in PBS containing 0.1% (w/v) sarkosyl (Fisher Scientific, UK) at pH 7.4.

2.16. DNA extraction from 10% (w/v) brain homogenates

DNA extraction was carried out using DNeasy blood and tissue kit (Qiagen, Germany) according to the manufacturer's specifications. Briefly, 250 µl of 10% (w/v) brain homogenate was pelleted at 17,000 g in a microfuge, the supernatant discarded and pellet resuspended in 180 µl buffer ATL and 20 µl of supplied PK. This mixture was then incubated at 56°C for 1 hour. 200 µl of buffer AL and 200 µl of ethanol were added and the mixture was briefly vortexed before being loaded onto the supplied mini spin columns and centrifuged at 6,000 g for 1 minute. The spin column was washed with 500 µl of

buffer AW1 and centrifugation at 6,000 g for 1 minute followed by 500 µl of buffer AW2 and centrifugation at 20,000 g for 3 minutes. The bound DNA was then eluted in 100 µl of buffer NE after incubated for 5 minutes on the column and centrifugation for 1 minute at 6,000 g. This elution process was repeated again with 100 µl of NE to ensure maximum DNA yield, in a total of 200 µl. The concentration of eluted DNA in the eluted fractions was then measured on a NanoDrop™ ND-8000 spectrophotometer (ThermoScientific, UK).

2.17. Amplification of extracted DNA by polymerase chain reaction (PCR)

DNA extracted from 10% (w/v) brain homogenates was amplified in PCR using a final amplification volume of 50 µl per reaction. 10 ng of DNA template was amplified using crimson *taq* buffer (New England Biolabs, UK) with 1 µl dNTPs (New England Biolabs, UK) 1 µl Taq polymerase (New England Biolabs, UK) and 10 µM of both forward and reverse primers. PCR primers were described by Colgan and colleagues and were designed to specifically amplify bovine or ovine DNA template (Colgan et al., 2001) (table 2.1). PCR tubes were sealed and placed in a thermocycler. Initially DNA was denatured at 94°C for 2 minutes. A further 1 minute of denaturation occurred and the temperature was lowered to the annealing temperature of 53°C for 30 seconds and then raised to the extension temperature of 72°C for 30 seconds. These PCR cycles were repeated up to 45 times before a final extension period of 5 minutes at 72°C and then samples were cooled to 4°C.

12.5 µl of PCR products were then analysed on a 2% (w/v) agarose gel (Sigma-Aldrich, UK) containing 3 µl NANCY-520 (Sigma-Aldrich) and ran at 100V for one hour in TAE buffer (VRW, UK). The gel was visualised in an ImageQuant LAS 3000 transilluminator (GE Healthcare, UK).

Table 2.1. Primers used in speciation PCR to test for the presence of ovine DNA in an excess of bovine DNA.

Primer ID	Species	Primer	Sequence	Source
fBov	Bovine	Forward	5’ GCCATATACTCTCCTTGG 3’	(Colgan et al., 2001)
rBov	Bovine	Reverse	5’ GTAGGCTTGGGAATAGTA 3’	(Colgan et al., 2001)
fOv	Ovine	Forward	5’ TTAAAGACTGAGAGCATGATA 3’	(Colgan et al. 2001)
rOv	Ovine	Reverse	5’ GAAAGAGGCAAATAGATTTTC 3’	(Colgan et al. 2001)

2.18. Taqman-based qPCR

DNA templates consisting of a total concentration of 10 µg were amplified using 10 µM of each primer and 5 µM of fluorophore tagged oligo (table 2.2). Primers and oligos were designed using NCBI BLAST tool against mitochondrial ATPase 8 gene, and were selected based on appropriate product length, high species specificity, and low primer self-reactivity. Templates and primers were diluted in TaqMan® environmental master mix 2.0 (Thermo Fisher Scientific, USA) which contained buffering agents and polymerase. The amplification cycle consisted of an initial denaturation step denaturation step at 95.0°C for 3 minutes, and the temperature was cycled between 65.5°C and 95.0°C for 30 seconds each, and the fluorescence at 520 nm was measured at the end of each annealing stage. The cycle was programmed and completed in a CFX96 thermocycler (Biorad, UK). Data analysis was conducted in the manufacturer's software Bio-Rad CFX manager 3.1.

Table 2.2. Primers and Fluorophore labelled oligos used in probe based qPCR

Oligo	Oligo ID	Species	Sequence	5' Fluorophore	3' Quencher
Forward primer	Ov For qPCR s3	Ovine	CCCCCTCGTTACCCTCATTG		
Reverse primer	Ov Rev qPCR s3	Ovine	CTGTTGGAGGGAGATGAGGC		
Labelled probe	Ov Probe qPCR s3	Ovine	CCCACATCAAACCGACTAGTCAACA	6FAM	BHQ1
Forward primer	Bov For qPCR s3	Bovine	GGTGACATGCCGCAACTAGA		
Reverse primer	Bov Rev qPCR s3	Bovine	GGGTTACGAGAGGGAGACCTA		
Labelled probe	Bov Probe qPCR s3	Bovine	ACACCCCTTGAGAAACAAAATGAACGA	6FAM	BHQ1

Chapter 3. Replacement of bioassays that quantify sPMCA products using *in vitro* amplification techniques

3.1. Introduction

The infectivity or infectious PrP^{Sc} load of a sample can give important information regarding disease progression in humans and animals, while informing best practises for industries and the medical profession regarding the decontamination of prion contaminated materials. The current gold standard for measuring infectivity is animal bioassays, which involves the transmission of PrP^{Sc} to defined hosts or a panel of hosts susceptible to disease, usually mice. End point dilution assays are used to determine the infectious (ID_{50}) or lethal (LD_{50}) doses for 50% of the animals inoculated (Stamp et al., 1959). While reliable and accurate, bioassays that measure infectivity require many animals to be infected with the dilution ranges of each TSE sample, are extremely expensive and take a large amount of time to complete. Therefore there has been interest in replacing these assays using prion *in vitro* amplification techniques which are much more rapid, attaining results in weeks rather than years, are far cheaper and require either a small amount of animal material or none at all, and never require the infection of animals with TSEs. We sought to merge the use of recombinant hamster PrP (rPrP) as substrate that can amplify a range of different scrapie strains with a method of quantifying replication efficiencies *in vitro* which is comparable to bioassay end point dilutions, to produce a standard assay capable of quantifying the infectivity of a range of scrapie strains. There were two main aims of this part of the study, firstly develop sPMCA conditions to produce sensitive and specific amplification of PrP^{Sc} using rPrP as the substrate and secondly produce a sPMCA method to reliably quantify infectivity which can be correlated to animal bioassay end point dilution values.

3.2. Results

3.2.1. Recombinant hamster PrP as a universal substrate for sPMCA

As a starting point to assess the suitability of recombinant hamster PrP^{C} as a universal substrate for the detection of PrP^{Sc} from a variety of scrapie sources, the substrate was applied to the amplification of 10% (w/v) brain homogenates from a pool of sheep from the Ripley flock (R01; figure 3.1). The Ripley flock has high incidence of natural scrapie, 100% of the animals with genotype of both VRQ/VRQ and AHQ/AHQ at amino acid positions 136, 154 and 171 in the PrP^{C} protein go on to develop disease (Ryder et al., 2004). sPMCA products were detected after 2 and 3 rounds when seeded with dilutions of R01 down to 500 and 50 ng respectively. The recombinant substrate lacks the PrP^{C} glycosylation states required to form the classical PrP^{Sc} triplicate, however the observed low molecular weight and duplicate bands of the sPMCA products seeded by R01 were characteristic of recombinant PMCA products when spiked with PrP^{Sc} (Atarashi et al., 2007). Amplification of substrate alone failed to generate sPMCA products when digested with 10 µg/ml PK (Figure 3.1).

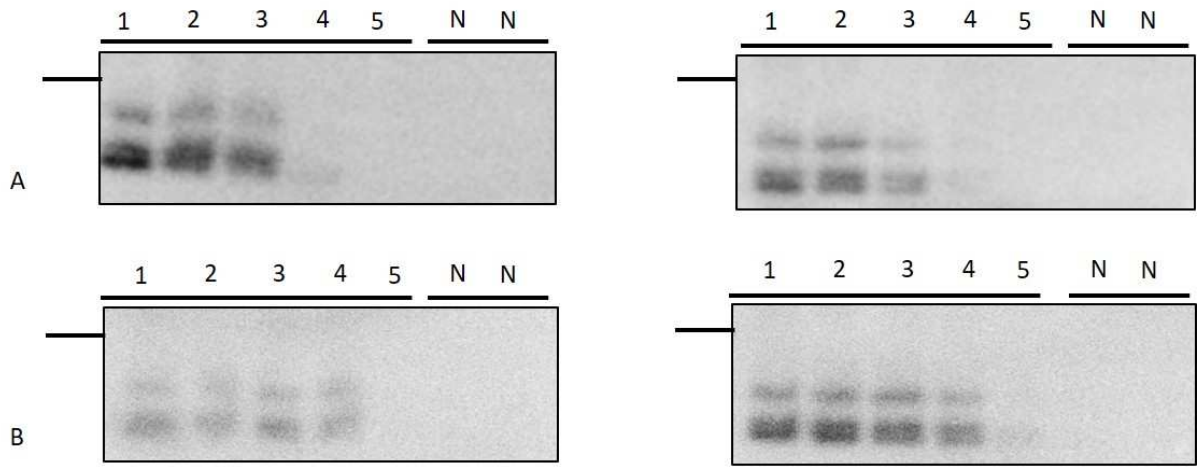


Figure 3.1. Amplification of scrapie PrP^{Sc} in sPMCA using recombinant hamster PrP as substrate. A dilution series of a scrapie positive brain homogenate (R01) was used to seed the misfolding of bacterially expressed hamster PrP. The substrate was seeded with 50,000, 5,000, 500, 50 or 5 ng of scrapie infected brain material (lanes 1 to 5 respectively) or contained only recombinant substrate (N) before being amplified for 2 (row **A**) or 3 (row **B**) rounds of sPMCA. The sPMCA products were digested with 10 µg/ml PK before western blotting and probing with SHa31 antibody. A 20 kDa marker is indicated to the left of each western blot.

Following the initial positive identification of PK-resistant sPMCA products when seeded with scrapie positive brain homogenate, which were not present in unseeded substrate only controls, a range of scrapie positive and TSE-free brain homogenates from different *PRNP* genotypes were subjected to sPMCA (table 3.1). Each of the 10 scrapie positive brain homogenates were able to seed positive sPMCA reactions after both 2 and 3 rounds of amplification (figure 3.2A and 3.2B respectively). Six of seven of the healthy, scrapie free, brain homogenates did not seed the conversion of recombinant substrate, however a positive reaction was observed after both 2 and 3 rounds of amplification when PG0664/09 was included in the sPMCA reactions.

Table 3.1. Ovine brain homogenates subjected to amplification in sPMCA with recombinant substrate.

Sample	TSE content	Genotype	Sample ID[a]
B26[b]	Scrapie	Not known	S1
PG1358/05	Scrapie	AHQ/ARQ	S2
PG0320/05	Scrapie	AHQ/ARQ	S3
PG1499/02	Scrapie	AHQ/VRQ	S4
PG0157/05	Scrapie	AHQ/VRQ	S5
PG0217/06	Scrapie	ARH/ARH	S6
PG0380/04	Scrapie	ARH/ARH	S7
PG1381/04	Scrapie	ARH/ARH	S8
PG0333/06	Scrapie	ARQ/ARH	S9
PG0781/05	Scrapie	ARQ/ARH	S10
PG2133/03	Scrapie	ARQ/ARQ	S11
PG1361/05	Scrapie	ARQ/VRQ	S12
PG0505/05	Scrapie	VRQ/ARH	S13
PG0376/06	Scrapie	VRQ/ARH	S14
PG0997/05	Scrapie	VRQ/ARH	S15
PG2069/03	Scrapie	VRQ/ARH	S16
PG1667/05	Scrapie	VRQ/ARR	S17
PG0730/05	Scrapie	VRQ/ARR	S18
PG0784/05	Scrapie	VRQ/ARR	S19
PG0794/05	Scrapie	Not known	S20
PG1418/08	Healthy	ARQ/ARQ	H1
PG1409/08	Healthy	ARR/ARR	H2
PG1410/02	Healthy	ARR/ARR	H3
DK03340702486	Healthy	VRQ/VRQ	H4
PG0649/09	Healthy	VRQ/VRQ	H5
PG0664/09	Healthy	VRQ/VRQ	H6
PG0665/09	Healthy	VRQ/VRQ	H7
PG0643/09	Healthy	VRQ/VRQ	H8
PG0670/09	Healthy	VRQ/VRQ	H9
PG0648/09	Healthy	VRQ/VRQ	H10
PG0643/09	Healthy	VRQ/VRQ	H11
PG0670/09	Healthy	VRQ/VRQ	H12
PG0648/09	Healthy	VRQ/VRQ	H13
PG1474/10	Healthy	AHQ/AHQ	H14
PG1376/10	Healthy	VRQ/VRQ	H15
PG1384/10	Healthy	AHQ/AHQ	H16
PG0143/12	Healthy	VRQ/VRQ	H17
PG0146/12	Healthy	VRQ/VRQ	H18
PG1418/08	Healthy	ARQ/ARQ	H19
PG1409/08	Healthy	ARR/ARR	H20

a) Designated sample identification number for western blotting.
b) Experimental scrapie pool. Individual animal ID and genotype unknown.

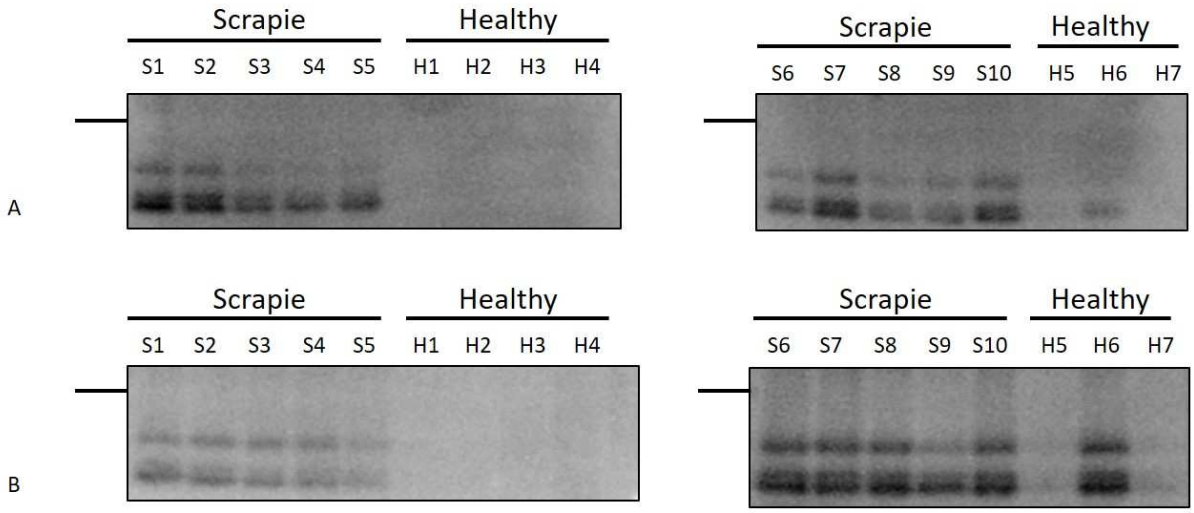


Figure 3.2. Recombinant sPMCA of scrapie positive and healthy 10% (w/v) ovine brain homogenates. Scrapie and healthy brain homogenates were amplified using recombinant hamster PrP for 2 (**A**) or 3 (**B**) rounds of sPMCA before digestion with 10 µg/ml PK. Digested proteins were separated by SDS-PAGE before transferral to PVDF membrane prior to probing with SHa31 primary antibody. 20 kDa makers are indicated to the left of western blots. Scrapie positive brain homogenates were B26, PG1358/05, PG0320/05, PG1499/02, PG0157/05, PG0217/06, PG0380/04, PG1381/04, PG0333/06 and PG0781/05 (S1-S10 respectively). Healthy brain homogenates were PG1418/08, PG1409/08, PG1410/02, YAH3014, PG0649/09, PG0664/09 and PG0665/09 (H1-H7 respectively).

3.2.1.1. Reducing the spontaneous generation of PrP^{Sc} during sPMCA amplification

In an attempt to prevent the formation of PK-resistant products induced in the amplification of healthy brain homogenates with recombinant hamster substrate, the volume of brain homogenate used as seed was decreased. The aim of this modification was to reduce the level of PrP amyloid fibrils or other co-factors in healthy brain homogenates which may be present and induce the formation of PK-resistant PrP. In previous recombinant sPMCA (figure 3.2) 50 µg of brain material was added to recombinant hamster substrate, in this case 5 and 0.5 µg of both healthy and scrapie infected brain material was used (figure 3.3). 5 µg seeds which were amplified for 2 rounds of sPMCA displayed 100% sensitivity in the 5 scrapie positive brain homogenates used as seeds, and 100% specificity as none of the healthy brain homogenates produced protease resistant products. There were also no PK-resistant products found in sPMCA reactions with substrate only. Seeds of only 0.5 µg spike also produced 100% sensitivity, as protease resistant products were present in at least one of each of the duplicates amplified. However the presence of protease resistant products was observed in one replicate which contained only recombinant substrate (N1) and a weak signal was also detected in one replicated seeded with healthy brain homogenate (H5).

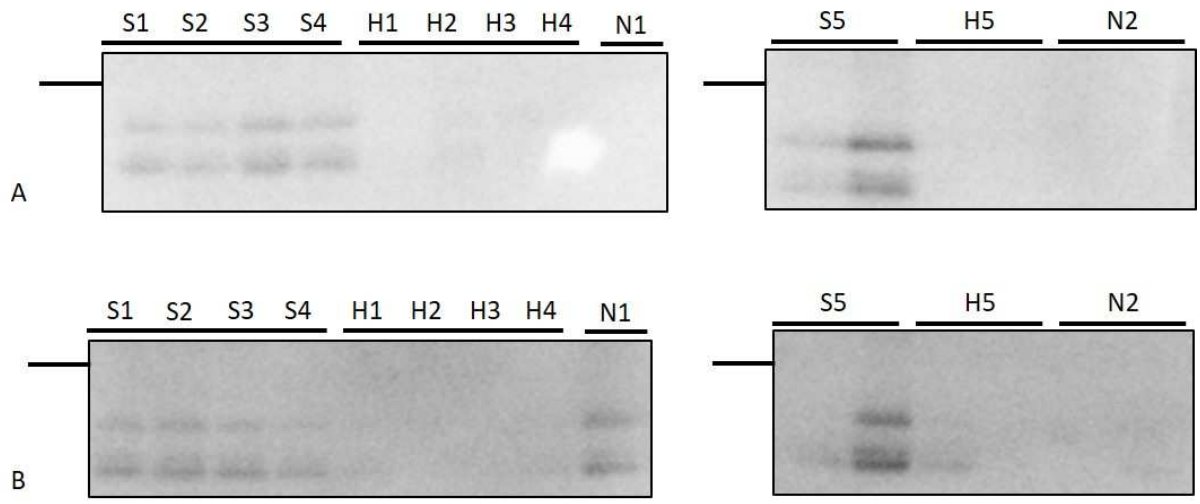


Figure 3.3. Recombinant sPMCA of diluted scrapie positive and healthy brain homogenate seeds. Recombinant hamster substrate was seeded with scrapie positive (S1-5) and healthy (H1-5) brain homogenates along with unseeded recombinant substrate (N1-2). 5 µg of brain spikes were amplified for 2 rounds of sPMCA (row **A**) and 0.5 µg amplified for 3 rounds (row **B**). sPMCA products were digested with 10 µg/ml PK and separated by SDS-PAGE before transferral to PVDF membrane and detection with SHa31 primary antibody. 20 kDa makers are indicated to the left of western blots. Scrapie positive brain homogenates were PG0217/06, PG0380/04, PG1381/04, PG0333/06 and PG0781/05 (S1-5 respectively). Healthy brain homogenates were PG1410/02, YAH3014, PG0649/09, PG0664/09 and PG0665/09 (H1-H5 respectively). Each sample was amplified in two separate sPMCA reactions, representative examples are shown for some of the samples.

The reduced level of non-specific protease resistant PrP when seeds of 5 µg brain material per sPMCA reaction were promising results. Therefore a larger cohort of both scrapie infected and healthy brain homogenates from a range of *PRNP* genotypes were used as seeds (figure 3.4). Again 100% (20/20) of the scrapie infected brain homogenates produced protease resistant PrP fragments after 2 rounds of amplification. However 12/20 of the replicates seeded with healthy brain homogenates produced PK resistant PrP fragments in at least one duplicate. Furthermore, 2 replicates that contained only recombinant hamster substrate also produced protease resistant PrP after 2 rounds of amplification.

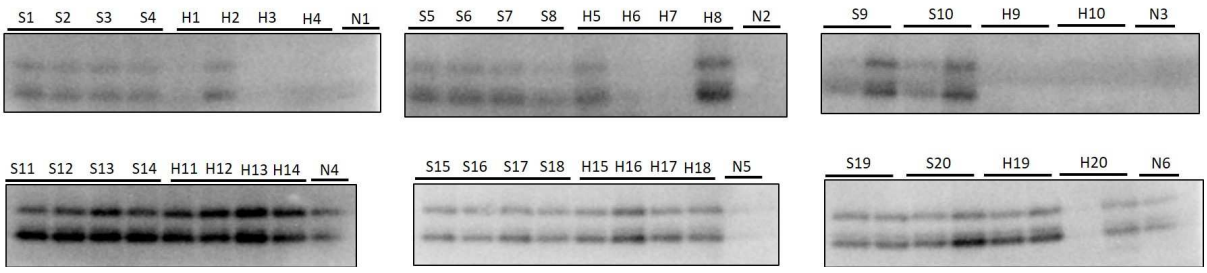


Figure 3.4. Recombinant sPMCA seeded with 5 µg of scrapie infected or healthy brain homogenates. Recombinant hamster substrate was seeded with scrapie infected (S1-20) and healthy (H1-20) brain homogenates and were subjected to 2 rounds of amplification along with substrate only controls (N1-6). sPMCA products were digested with 10 µg/ml PK and separated by SDS-PAGE before

transferral to PVDF membrane and detection with SHa31 primary antibody. Scrapie positive and healthy brain homogenates are detailed in table 3.1.

3.2.1.1. Use of additives to reduce non-specific formation of protease resistant PrP

Two additives were included in sPMCA reactions to in an attempt to reduce the non-specific formation of protease resistant PrP, which were digitonin and α-crystallin. Previously, digitonin has been added to sPMCA reaction to prevent the formation of protease resistant PrP^C (Murayama et al. 2007). It is possible the formation of protease resistant PrP^C could be seeding the non-specific formation of protease resistant fragments that were observed in reactions containing substrate only and those seeded with healthy brain homogenates. Digitonin was added to the sPMCA reaction buffer to final concentrations of both 0.05 and 0.005% (w/v) which was spiked with 5 µg of either healthy or scrapie-infected brain material and amplified for 3 rounds (figure 3.5). Reactions seeded with scrapie positive brain material induced the conversion of protease resistant sPMCA products. However amplification of healthy brain homogenates at rounds 2 and 3 also induced PK-resistant PrP at both 0.05 and 0.005% (w/v) digitonin. In reactions seeded with both healthy and scrapie infected brain tissues protease resistant PrP can be observed in the sPMCA products at round 3.

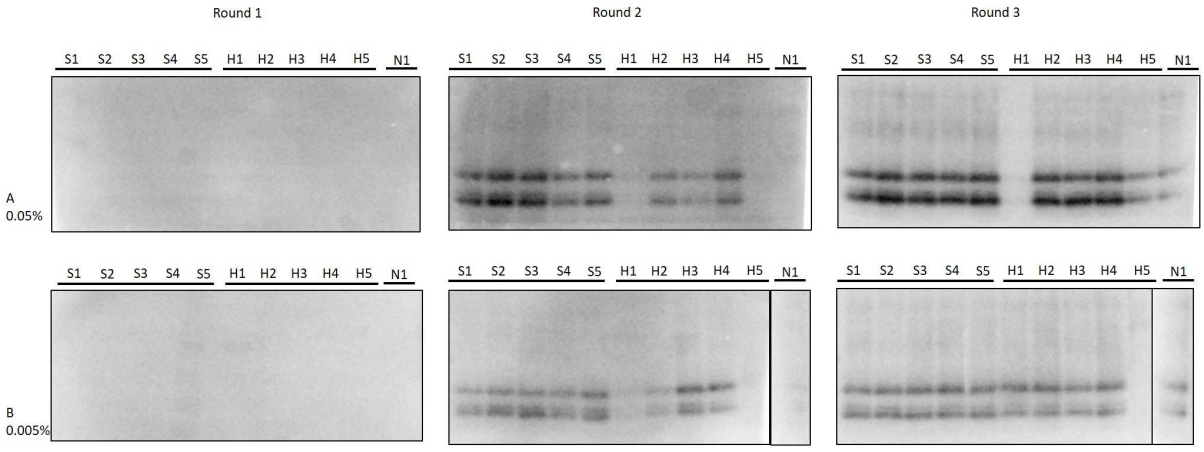


Figure 3.5. Addition of digitonin to recombinant sPMCA reactions. Scrapie infected (S1-5) and healthy (H1-5) brain homogenates were used to seed sPMCA reactions using 5 µg of brain material and were amplified along with substrate-only reactions for 3 rounds of amplification. Digitonin was added to sPMCA reactions at 0.05% (**A**) or 0.005% (**B**). sPMCA products were digested with 10 µg/ml PK and separated by SDS-PAGE before transferral to PVDF membrane and detection with SHa31 primary antibody. Scrapie positive brain homogenates were PG0217/06, PG0380/04, PG1381/04, PG0333/06 and PG0781/05 (S1-5). Healthy brain homogenates; PG1418/08, PG0649/09, PG0664/09, PG0665/09 and PG1384/10 (H1-5).

α-crystallin from bovine eye lens has been used as a sPMCA additive and demonstrated potential by altering the amplification efficiency of vCJD PrP^{Sc} in sPMCA (Barria, 2014). At low concentrations (0.1 and 0.01 μg/ml) α-crystallin increased the amplification efficiency of vCJD PrP^{Sc}, however at higher concentrations (10 μg/ml) an inhibitory effect in amplification was observed. Therefore it was theorised that there may be a concentration of α-crystallin which could be used as an additive which could prevent the *in vitro* generation of protease resistant PrP. Two scrapie infected brain homogenates (PG0380/09 and PG1381/04) and two brain homogenates from healthy animals (PG0649/09 and PG0664/09) which have demonstrated a high rate of forming protease resistant products were amplified in sPMCA with α-crystallin additive (figure 3.6). Protease resistant PrP was observed when reactions were spiked with scrapie-infected brain homogenates in the presence and absence of α-crystallin, though protein bands appeared more intense when α-crystallin was present. Protease resistant PrP^{C} was also found in at least one replicate of healthy brain homogenate seeded sPMCA in the absence and at every concentration of α-crystallin. Healthy brains without the addition of α-crystallin were the only replicates clearly without the *de novo* formation of protease resistant PrP. There appeared to be no correlation between the rate of spontaneous formation of protease resistant PrP and the concentration of α-crystallin included in the sPMCA reactions.

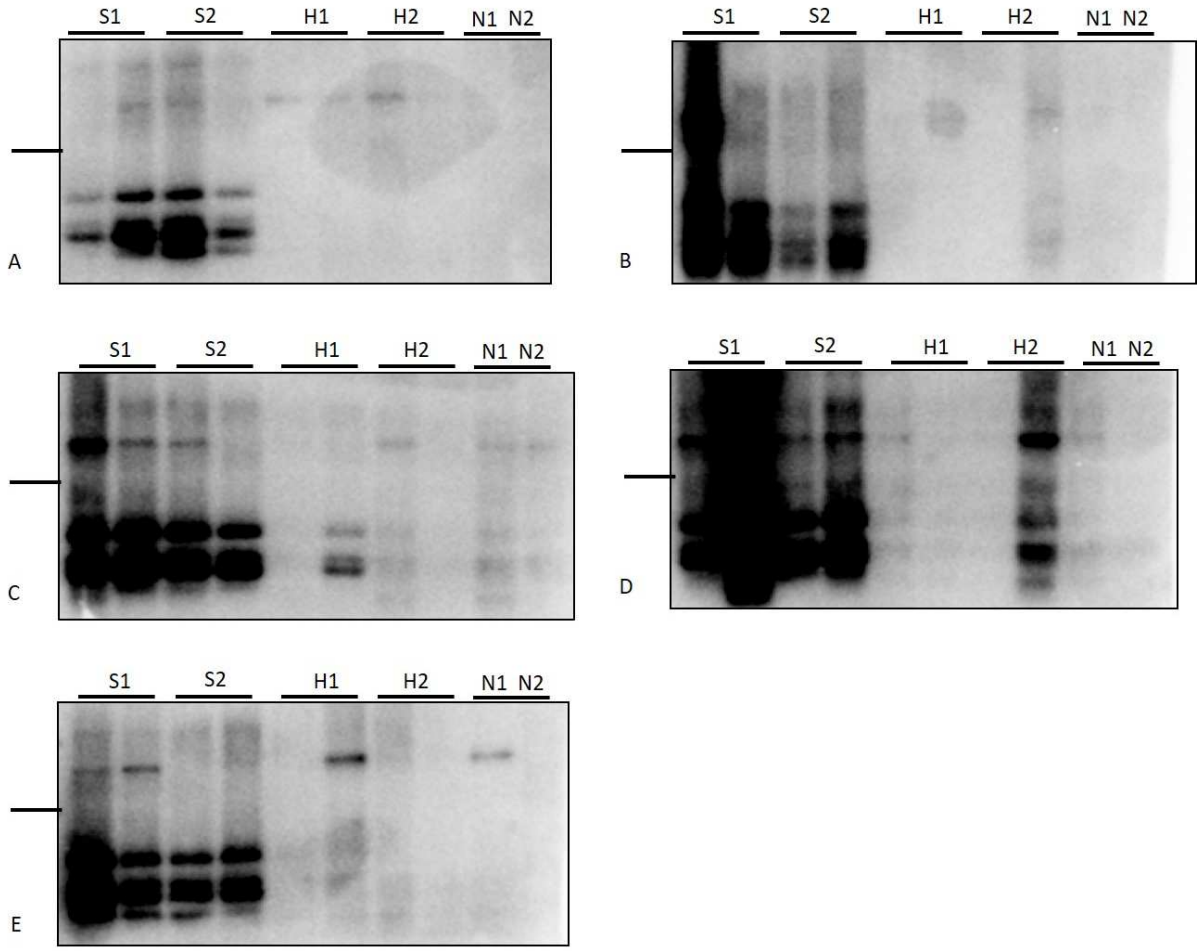


Figure 3.6. ***In vitro*** **amplification of scrapie and healthy brains with the addition of α-crystallin.** 5 µg of scrapie infected and healthy brain tissue was amplified with either no α-crystallin (**A**), or at 0.1 µg/ml (**B**), 1 µg/ml (**C**), 10 µg/ml (**D**) and 100 µg/ml (**E**). sPMCA products were digested with 10 µg/ml PK after two rounds of amplification and separated by SDS-PAGE before transferral to PVDF membrane and detection with SHa31 primary antibody. Scrapie positive brain homogenates were PG0380/09 and PG1381/04 (S1 and S2). Healthy brain homogenates; PG0649/09 and PG0664/09 (H1 and H2). 20 kDa marker is indicated to the left of each blot.

3.2.2. Application of quantitative sPMCA to BSE for the estimation of brain material in reaction spikes

Quantitative sPMCA (qPMCA) is a method developed to measure the *in vitro* amplification efficiency of the seed which may in turn be representative of the *in vivo* infectivity described by animal bioassay. In our hands recombinant hamster PrP is an unsuitable substrate for sPMCA and therefore not suitable for a standardised quantification assay, there is still many applications of an *in vitro* alternative to bioassays which utilise brain derived PrP^C as substrate rather than rPrP. To develop a sensitive and robust amplification method based on amplification in 10% (w/v) brain homogenates rather than recombinant PrP sPMCA, conditions must be trialled in order to find optimal conditions for amplification.

Initially an isolate of bovine BSE SE1946/0002 was amplified in bovine substrate for 5 rounds of sPMCA. To find the optimum spike for sPMCA the BSE infected brain content was amplified using a 10-fold dilution series of spike from 100 µg to 10 pg. The sPMCA products at each round of amplification were digested with 50 µg/ml and loaded onto nitrocellulose membranes before being probed with SHa31 primary antibody. Dot blotting was used in place of SDS-PAGE and western blotting because it is has a relatively high throughput in comparison and effective qPMCA relies on a large numbers of samples. The BSE isolate showed very poor amplification, PrP^{Sc} was only detected in reactions seeded with 100 and 10 µg of brain (figure 3.7).

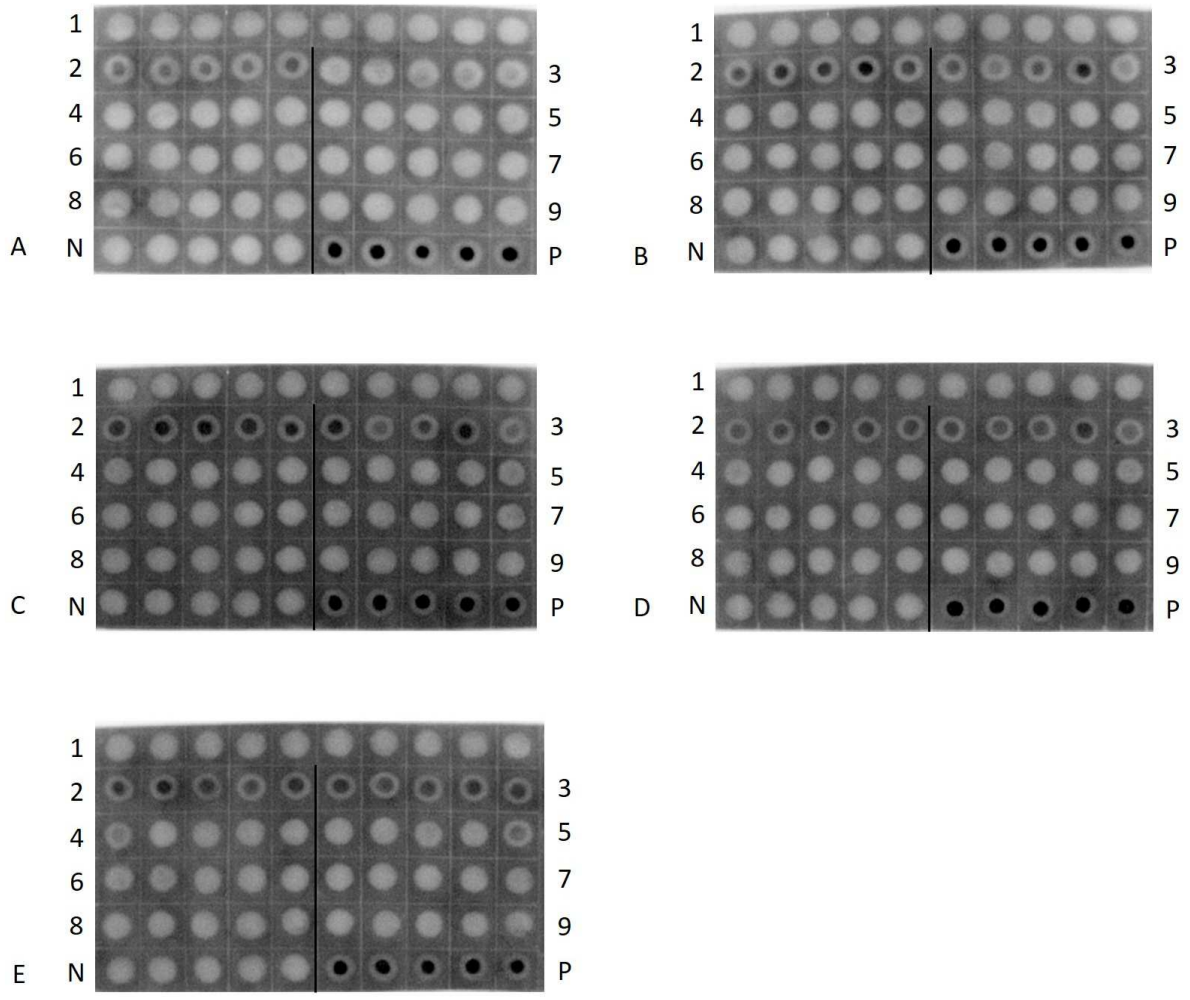


Figure 3.7. Limit of detection of BSE in qPMCA using bovine substrate. Bovine BSE SE1946/0002 was amplified for 5 rounds in bovine substrate. sPMCA reactions contained no spike (**1**) or 100 µg (**2**), 10 µg (**3**), 1 µg (**4**), 100 ng (**5**), 10 ng (**6**), 1 ng (**7**), 100 pg (**8**) and 10 pg (**9**) of SE1946/0002. The sPMCA products of each round of amplification were analysed, blot **A**=round 1, **B**=round 2, **C**=round 3, **D**=round 4, **E**=round 5. After each round, qPMCA products were digested with 50 µg/ml PK before being loaded onto nitrocellulose membrane along with BSE positive (**P**) and negative unamplified substrate (**N**) blotting controls and probed with SHa31 primary antibody.

3.2.2.1. Ovine AHQ/AHQ substrate as a substrate for qPMCA.

As the overall detection of BSE was low in the initial limit of detection experiment, it was repeated in ovine AHQ/AHQ substrate which is an efficient substrate for the amplification of bovine BSE in sPMCA (Chapter 7.2.1). To further increase the ability of the qPMCA to quantify the seeding ability of unknown samples the dilution series was contracted to a 5-fold decrease per step starting at a spike of 1 mg of brain tissue and decreasing until 12.8 ng. These modifications produced more positive signals in dot-blotting which enabled the creation of a standard curve (figures 3.8 and 3.9). Using the signal of the negative blotting controls to generate a background cut off value for each dot bot, which was the mean plus 5 standard deviations, the signal of each qPMCA replicate was measured and designated as a positive or negative signal. Positive signals were scored depending on the round at which they were analysed, a positive signal in round one scored 5, each round the score decreased by 1 for a positive results, and therefore a positive signal at round 5 scores a replication value of 1. The sum of the replication scores for each round were summed and plotted against the brain content in the spike for points down to 320 ng (figure 3.9). There was a strong correlation between the replication efficiency and the brain content of the spike, which produced an R^2 value of 0.9072 in non-linear correlation using a semi-log line fit in GraphPad Prism.

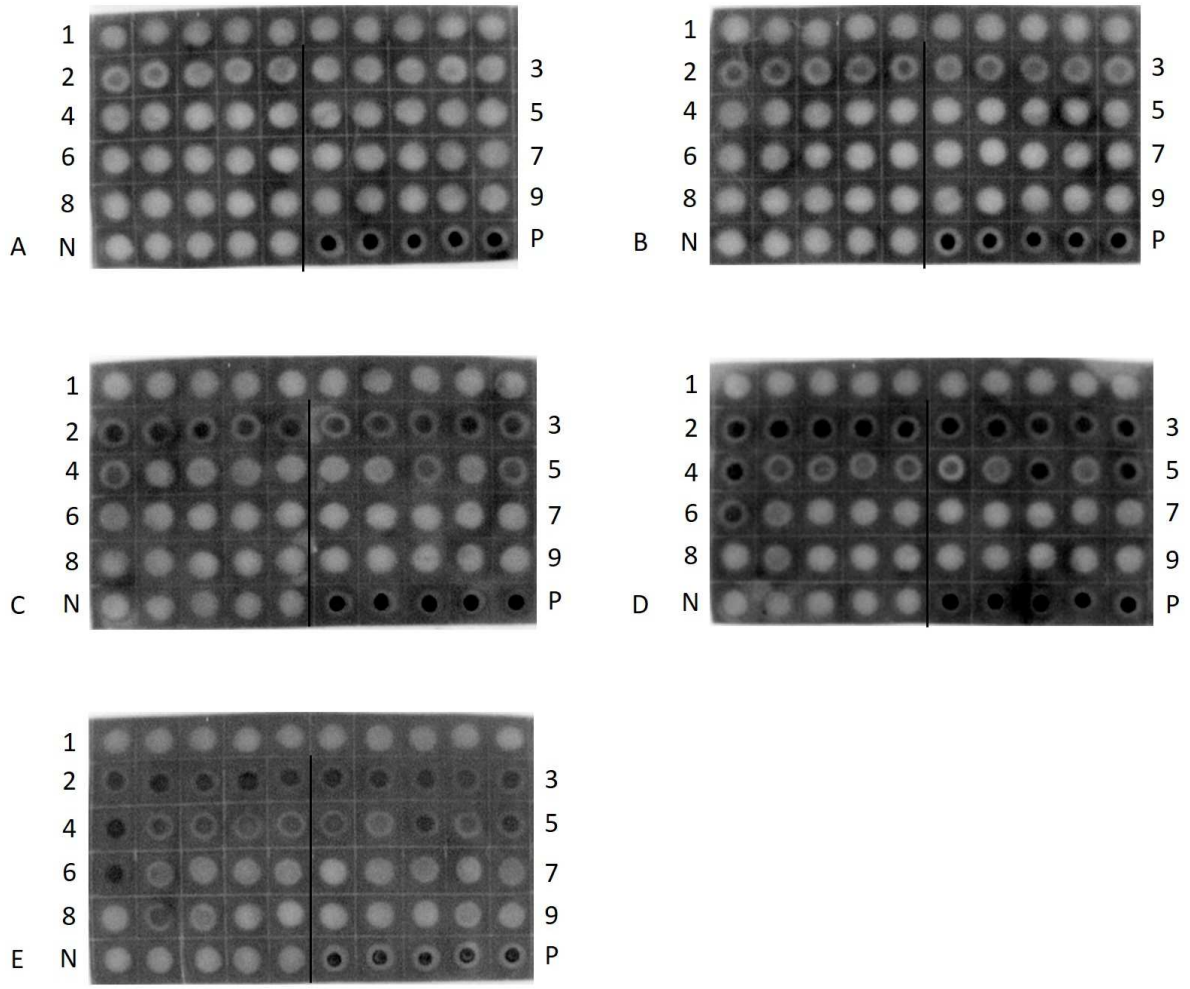


Figure 3.8. qPMCA limit of detection for BSE using ovine AHQ/AHQ substrate. Bovine BSE SE1946/0002 was amplified for 5 rounds in ovine AHQ/AHQ substrate. sPMCA reactions contained no spike (1) or 1 mg (2), 200 µg (3), 40 µg (4), 8 µg (5), 1.6 µg (6), 320 ng (7), 64 ng (8) and 12.8 ng (9) of SE1946/0002. The sPMCA products of each round of amplification were analysed per blot **A**=round 1, **B**=round 2, **C**=round 3, **D**=round 4, **E**=round 5. After each round qPMCA products were digested with 50 µg/ml PK before being loaded onto nitrocellulose membrane along with BSE positive (P) and negative unamplified substrate (N) blotting controls and probed with SHa31 primary antibody.

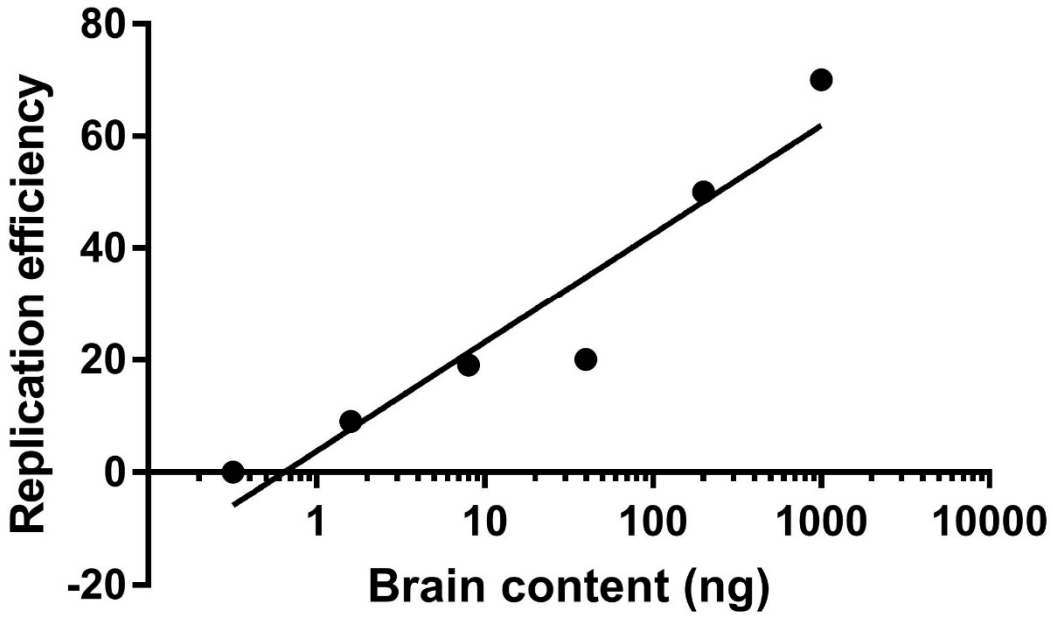


Figure 3.9. Correlation between BSE brain content of spike and amplification efficiency after sPMCA. The brain content of the sPMCA spikes, between 1 mg and 320 ng, was plotted against the replication efficiency of sPMCA replicates. Replication efficiency for each reaction was scored out of 15, a positive signal after one round of qPMCA scored 5, which decreased by 1 in each subsequent round (i.e. a reaction positive at all rounds had a total replication efficiency of 5+4+3+2+1 = 15; 5 replicates were carried out for each spike level and so each has a possible total replication efficiency of 75). Positive signal was assessed using a cut of value generated from the mean plus 5 standard deviations of the negative blotting controls. The standard curve and subsequent interpolation was conducted using the semi-log non-linear line function in GraphPad Prism, which had an R^2 value of 0.9072.

3.2.2.1. Application of qPMCA to estimate the brain content of BSE reaction seeds

To assess the ability of qPMCA as a method to replace bioassays that measure infectivity of TSEs the qPMCA of SE1946/0002 was repeated with dilutions (test samples) read off the standard curve. A standard curve was repeated and two extra sample sets at 40 and 1.6 µg spike levels were included as test samples to generate a replication value which could be used to estimate the brain content used to seed the qPMCA reactions (figure 3.10). Although overall amplification appeared less efficient than in the initial experiment, plotting amplification efficiency against brain content for replicates down to 1.6 µg (below this level of brain content amplification appeared unpredictable) allowed the brain content of the 40 and 1.6 µg spikes to be estimated from the replication efficiency as 36.5 and 2.8 µg respectively. This estimation was calculated in GraphPad Prism using the non-liner regression semi-log line function which had an R^2 value of 0.9311 (figure 3.11).

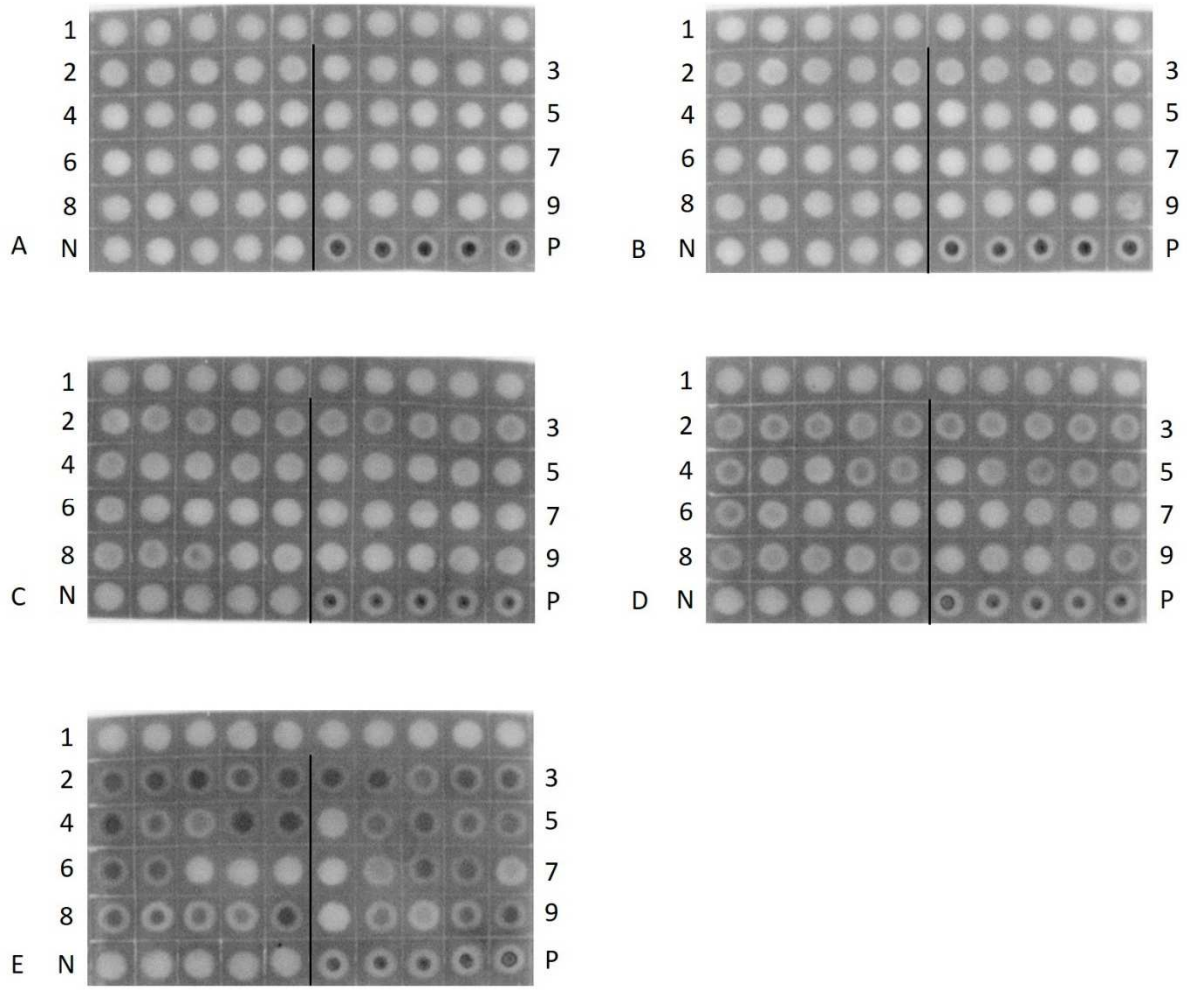


Figure 3.10. Estimation of BSE brain content of spike by amplification efficiency correlation after qPMCA. Bovine BSE SE1946/0002 was amplified for 5 rounds in ovine AHQ/AHQ substrate. sPMCA reactions contained no spike (1) or 1 mg (2), 200 µg (3), 40 µg (4), 8 µg (5), 1.6 µg (6), 320 ng (7) of brain material. Further replicates at 40 µg (8) and 1.6 ng (9) of SE1946/0002 were included to estimate brain content from the amplification efficiency. The sPMCA products of each round of amplification were analysed per blot **A**=round 1, **B**=round 2, **C**=round 3, **D**=round 4, **E**=round 5. After each round qPMCA products were digested with 50 µg/ml PK before being loaded onto nitrocellulose membrane along with BSE positive (P) and negative unamplified substrate (N) blotting controls and probed with SHa31 primary antibody.

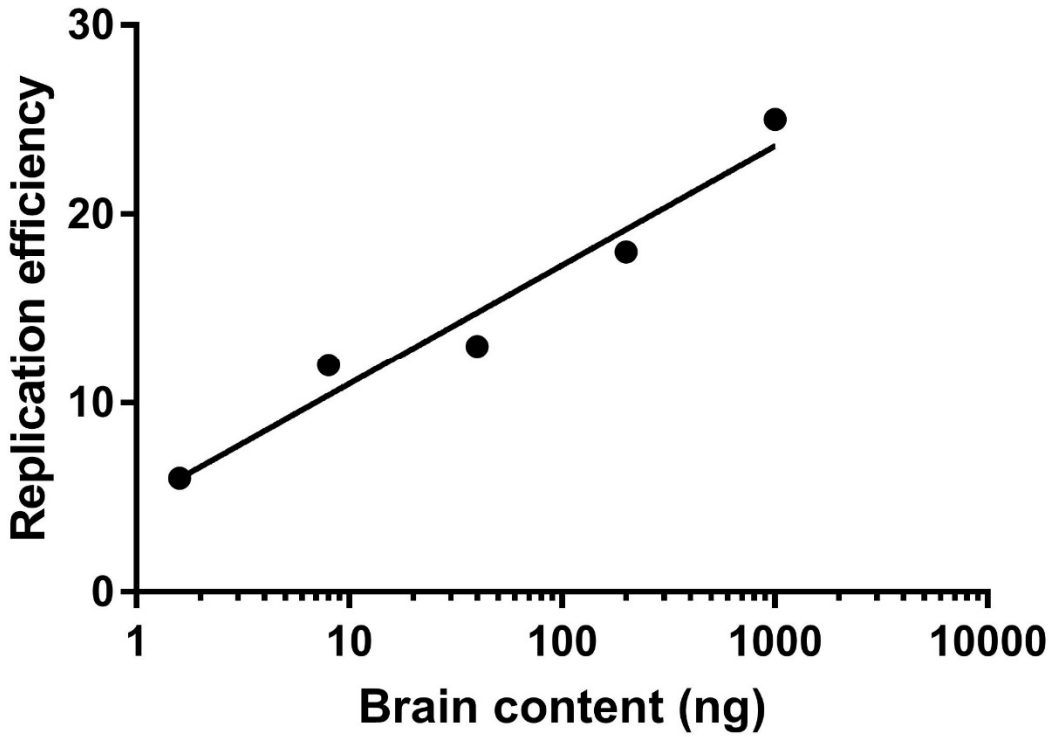


Figure 3.11. Estimation of BSE brain content of spike by amplification efficiency correlation after qPMCA. The brain content of the sPMCA spikes, between 1 mg and 1.6 µg, was plotted against the replication efficiency of sPMCA replicates. Replication efficiency for each reaction was scored out of 15, a positive signal after one round of qPMCA scored 5, which decreased by 1 in each subsequent round (i.e. a reaction positive at all rounds had a total replication efficiency of 5+4+3+2+1 = 15; 5 replicates were carried out for each spike level and so each has a possible total replication efficiency of 75). Positive signal was assessed using a cut of value generated from the mean plus 5 standard deviations of the negative blotting controls. The standard curve and subsequent interpolation was conducted using the semi-log non-linear line function in GraphPad Prism which had an R^2 value of 0.9311.

3.2.2.2. Reproducibility of qPMCA to quantify brain content of PrP^{Sc} spikes.

The previous data demonstrated that qPMCA estimation of brain material was within 8.75 and 75% of the actual values of the two points used (figures 3.10 and 3.11). To assess the reproducibility of this estimation another standard curve of SE1946/0002 was amplified in qPMCA with 2 replicate sample sets of both 40 and 1.6 µg of brain tissue. Over the course of the qPMCA the intensity of positive sample signals appeared to develop in later rounds than previously observed (figure 3.12). However when the cut off value (mean plus three standard deviations of negative blotting controls) was generated and applied to each round of amplification a fairly strong correlation was present between the brain content in the spike and the nominal replication value (figure 3.13). Non-linear regression analysis in GraphPad Prism using semi-log line function produced a best fit line with an R^2 value of

0.7971, while the correlation between replication efficiency and spike brain content was lower than previously analysed (figures 3.9 and 3.11) it was still used to estimate the brain content of reactions seeded with 40 and 1.6 µg BSE brain. The two sample sets seeded with 40 µg tissue were estimated to contain 32 and 12 µg, and the qPMCA estimate the brain content or samples seeded with 1.6 µg were both 0.98 µg (for summary see table 3.2).

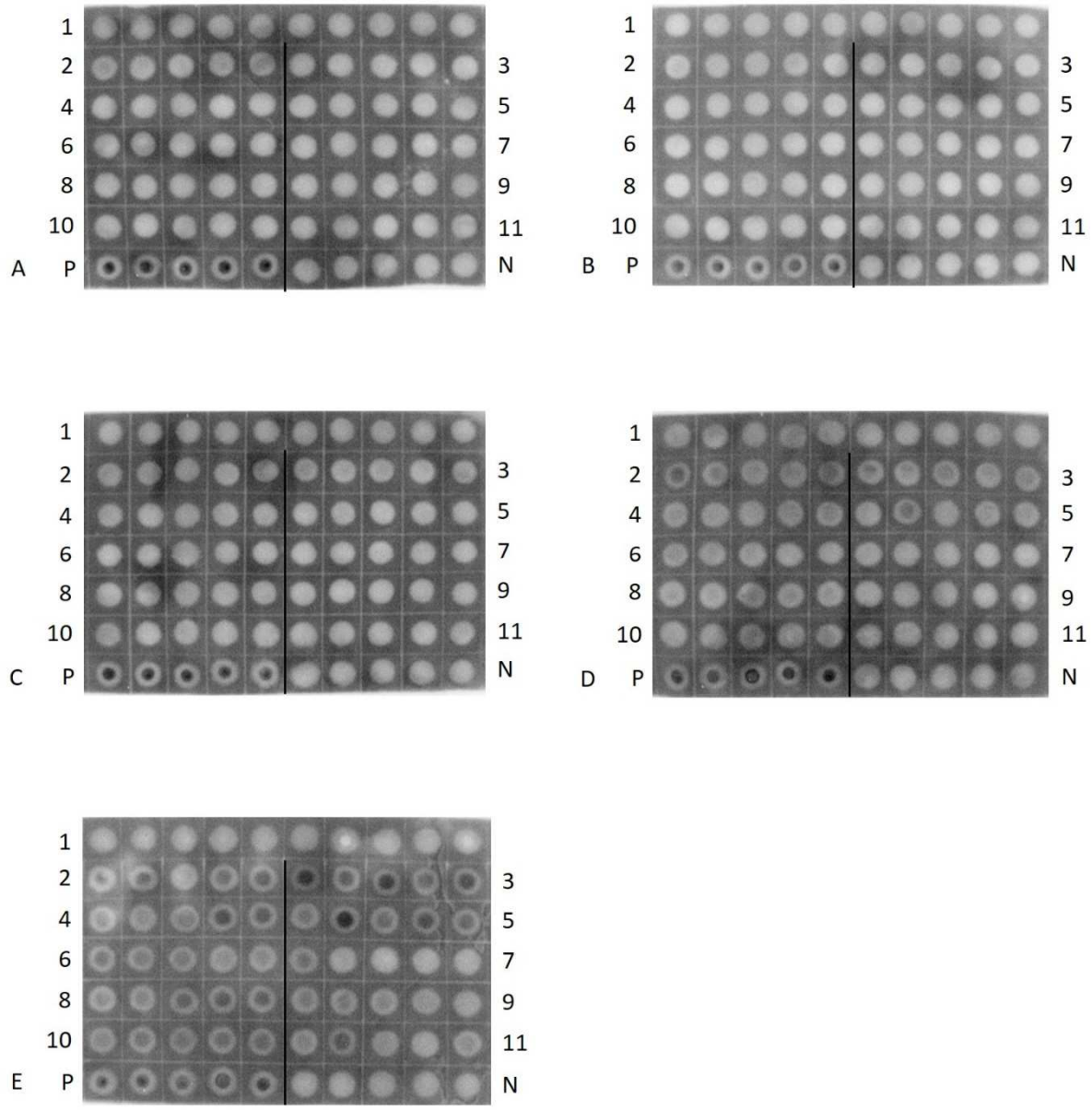


Figure 3.12. Dot blots of qPMCA to find reproducibility of spike brain content estimation. Bovine BSE SE1946/0002 was amplified for 5 rounds in ovine AHQ/AHQ substrate. sPMCA reactions contained no spike (1) or 1 mg (2), 200 µg (3), 40 µg (4), 8 µg (5), 1.6 µg (6), 320 ng (7) of brain material. Further replicates at 40 µg (8 and 10) and 1.6 ng (9 and 11) of SE1946/0002 were included to estimate brain content from the amplification efficiency. The sPMCA products of each round of amplification were analysed per blot **A**=round 1, **B**=round 2, **C**=round 3, **D**=round 4, **E**=round 5. After each round qPMCA products were digested with 50 µg/ml PK before being loaded onto nitrocellulose membrane along

with BSE positive (P) and negative unamplified substrate (N) blotting controls and probed with SHa31 primary antibody.

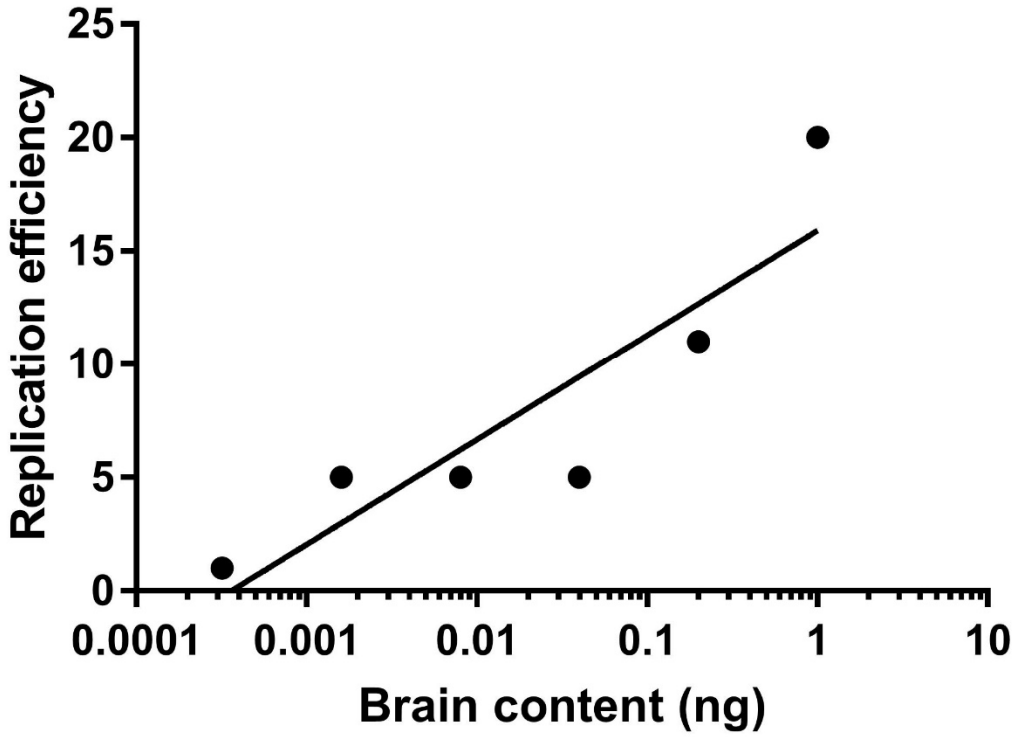


Figure 3.13. Correlation of replication efficiency and brain content of the qPMCA spike of BSE standard curve. The replication efficiency of each qPMCA product was plotted against the brain content of the spikes used to seed the reaction. Replication efficiency for each reaction was scored out of 15, a positive signal after one round of qPMCA scored 5, which decreased by 1 in each subsequent round (i.e. a reaction positive at all rounds had a total replication efficiency of 5+4+3+2+1 = 15; 5 replicates were carried out for each spike level and so each has a possible total replication efficiency of 75). Positive signal was assessed using a cut of value generated from the mean plus 5 standard deviations of the negative blotting controls. The standard curve and subsequent interpolation was conducted using the semi-log non-linear line function in GraphPad Prism which had an R^2 value of 0.7971.

Table 3.2. Summary of estimations of brain content in qPMCA using replication efficiency correlation

Actual brain content[a] (µg)	Estimated brain content[b] (µg)			Average	Standard deviation
40	36.43	32.00	11.81	26.75	10.72
1.6	2.82	0.98	0.98	1.59	0.87

a) Amount of BSE infected brain used to seed qPMCA reaction
b) Amount of BSE estimate to seed reaction using replication efficiency correlation to standard curve

3.2.2.3. Correlation of BSE isolate PMCA replication efficiency to bioassay generated quantitation data.

Correlation of replication efficiency and brain material for a single BSE isolate has demonstrated that qPMCA can estimate the quantity of brain material with a good level of accuracy, between 40% and 1% error depending on seed used (table 3.2). For this technique to reliably estimate BSE content from different sources the replication efficiency must demonstrate strong correlation with bioassay from a range of isolates. Therefore 100 µg of brain material from 4 BSE isolates that had been assayed in bioassays were amplified in sPMCA (figure 3.14), SE1945/0035, SE1762/0003, SE1762/0013, and SE1929/0749. After each round the sPMCA products were digested and dot blotted before being analysed by densitometry to determine amplification efficiencies. The amplification efficiencies of the BSE isolates did not correlate with the LD_{50} value generated from bioassay in RIII mice (table 3.3). SE1762/0013 had the highest replication efficiency (33) yet in bioassay was judged to be the least infectious to RIII mice ($10^{3.7}$ LD_{50}/ml). Sample SE1929/0749 had the lowest replication efficiency (16) but in mouse bioassay had comparable infectivity ($10^{4.15}$ LD_{50}/ml) to SE1945/0035 ($10^{4.24}$ LD_{50}/ml) and SE1762/0003 ($10^{4.65}$ LD_{50}/ml), both of which had larger replication efficiency values. Together these results indicate that the PrP^{Sc} efficiency of replication in this sPMCA does not correlate with the infectious titres obtained from bioassay in RIII mice.

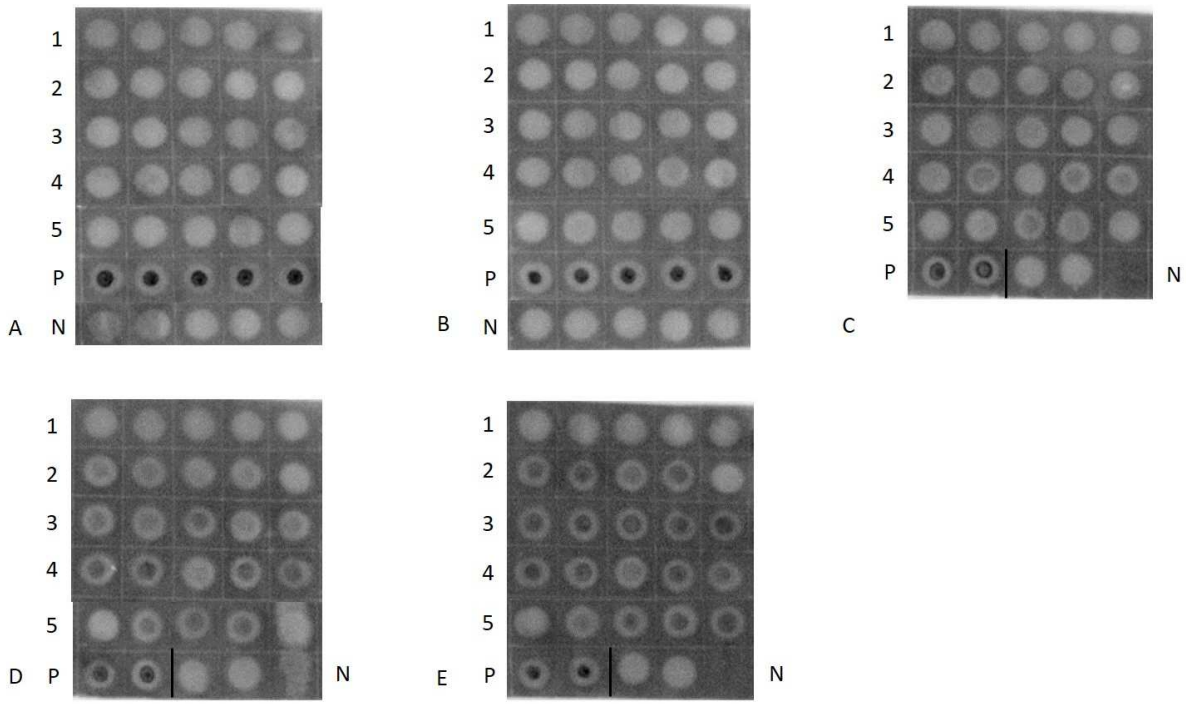


Figure 3.14. Amplification of BSE isolates which had previously been assayed in mouse bioassay. Five Bovine BSE isolates were amplified for 5 rounds in ovine AHQ/AHQ substrate. sPMCA reactions contained no brain material (**1**) or 100 µg of BSE brain material from isolates SE1945/0035 (**2**), SE1762/0003 (**3**), SE1762/0013 (**4**) and SE1929/0749 (**5**). The sPMCA products of each round of amplification were analysed per blot **A**=round 1, **B**=round 2, **C**=round 3, **D**=round 4, **E**=round 5. After each round qPMCA products were digested with 50 µg/ml PK before being loaded onto nitrocellulose membrane along with BSE positive (P) and negative unamplified substrate (N) blotting controls and probed with SHa31 primary antibody.

Table 3.3. Comparison of sPMCA derived replication efficiencies to bioassay LD_{50}/ml value

BSE Isolate	Replication efficiency	LD_{50}/ml
SE1945/0035	24	$10^{4.24}$
SE1762/0003	30	$10^{4.65}$
SE1762/0013	33	$10^{3.7}$
SE1929/0749	16	$10^{4.15}$

3.3. Discussion

3.3.1. Recombinant PMCA

The use of bacterially expressed recombinant PrP as a substrate for *in vitro* amplifications of prions in PMCA is attractive as it enables the generation of consistent substrate for sPMCA reactions, unlike brain derived 10% (w/v) homogenates which may vary between animals due to the diversity of unknown co-factors, inhibitors or enhancers in the homogenate. Furthermore, bacterially expressed

proteins are relatively easy to genetically manipulate allowing for directed changes to the structure of the PrP and experimentally tailored substrates with molecular tags. Recombinant proteins can also be much more easily purified and concentrated, because of this the estimates of recombinant PrP content are much more accurate and straight forward than those of PrP^{C} in brain homogenates, potentially enabling better control of sPMCA reaction conditions.

The use of recombinant PrP produced in *Escherichia coli* as substrate for misfolding of PrP^{Sc} in sPMCA was first described by Atarashi and colleagues (Atarashi et al., 2007). They used bacterially expressed hamster PrP as a substrate for the conversion of hamster adapted scrapie 263K. PrP^{Sc} could be detected from scrapie infected brain homogenates and CSF, in the brain homogenates PrP^{Sc} could be detected when seeded with only 50 ag of brain material which the authors estimated to contain approximately 1000 PrP^{Sc} molecules. The sPMCA products did not display typical molecular weights of the classical PrP^{Sc} triplet due to the lack of glycosylation of recombinant PrP. The recombinant sPMCA products had multiple protein bands in western blotting, the most prominent of which was ~17 kDa and resembled de-glycosylated PrP^{Sc}.

Recombinant PMCA has also been carried out with recombinant PrP ovine VRQ and ARQ and cervid QGY (cervid amino acid positions 95, 96 and 216) proteins that were expressed from a baculovirus vector in insect cells with mammalian N-glycosyltransferase (Faburay et al., 2014). The advantage of this expression system over bacterial expression is that the recombinant proteins are subjected to post-translational modifications that result in a GPI-anchor and glycosylation at the three glycosylation sites, so more closely resemble mammalian PrP^{C}. Baculovirus expressed recombinant PrP displayed the three glycosylation states of PrP^{C} which was partially maintained when ovine and cervid recombinant PrP was seeded with scrapie or CWD PrP^{Sc} respectively and amplified in sPMCA. The recombinant sPMCA was utilised to assess effect of misfolding promoters during *in vitro* amplification, such as RNA molecules and manganese ions.

Recombinant PrP substrates have been used to amplify a range of different TSEs including scrapie, chronic wasting disease, BSE and CJD in PMCA (Atarashi et al. 2007; Panza et al. 2008; Faburay et al. 2014; Fujihara et al. 2009). Wild-type hamsters are susceptible to infection by many different TSEs, including scrapie, TME, Classical BSE, H-type BSE, and L-type BSE, and are more susceptible to scrapie infections than mice lines used in bioassay, therefore highly permissible *in vivo* conversion of hamster PrP^{C} identified recombinant hamster PrP as a candidate for a universal substrate. (Marsh et al. 1975; Nicot et al. 2011; Thomzig et al. 2004; Shu et al. 2011). The recombinant sPMCA method described above was designed to be the first stage of a drive to produce a universal substrate, which could be applied to standardise a qPMCA methodology that could measure and compare the infective titre of PrP^{Sc} from different scrapie sources. While preliminary amplification of scrapie PrP^{Sc} in recombinant

hamster substrate appeared promising it became clear that PrP^{C} or co-factors from scrapie free brain homogenates were producing false positive results, despite robust liquid handling controls to prevent cross contamination of samples. Liquid handling controls included exclusive use of filter pipette tips, sealing negative controls before opening scrapie positive tubes, ensuring only one sPMCA tube was open at any one time, changing gloves between samples and regular decontamination of plastic-ware. The tendency of bacterially expressed recombinant PrP^{Sc} to form β-sheet fibrils when exposed to shaking, in the absence of any co-factors, which are protease resistant has recently been demonstrated (Ladner-Keay et al., 2014). It is feasible that in sPMCA a similar event may be taking place in which the sonication stage may be inducing the formation of *de novo* PK-resistant amyloid fibrils. The total false positive rate for healthy brain homogenates spiked sPMCA replicates was 29% (39/135), but the rate in replicates containing only rPrP was 15% (6/39) The increased rate of this non-specific formation of PK-resistant recombinant PrP in PMCA seeded with scrapie free brain homogenates may be due to co-factors present in the homogenates. Urea, phospholipids, nucleic acids and pH changes have previously been shown to induce formation of PK-resistant β-sheet fibrils (Baskakov et al., 2002; Deleault et al., 2003; Wang et al., 2007). Recombinant hamster PrP has previously been shown to spontaneously convert into PK-resistant fragments during sPMCA and this was reduced by including 0.1% Triton X-100 in the reaction buffer (Atarashi et al., 2007). Whilst spontaneous PK-resistant products still occurred, the authors were able to distinguish the PrP^{Sc} seeded and spontaneous PK-resistant recombinant PrP primarily through the molecular weights of the products, the non-specific PrP lacked the prominent 17 kDa protein band. However this is in contrast with the results from the sPMCA described here and also the generation of PK-resistant products through shaking, in both cases recombinant *de novo* and PrP^{Sc} seeded recombinant PrP displayed identical molecular weights (Ladner-Keay et al., 2014).

Two sPMCA additives were trialled to reduce the formation of this protease resistant PrP, digitonin and α-crystallin. Digitonin has ablated the formation of protease resistant PrP^{C} from mouse brain homogenates used as substrate for sPMCA, by addition at 0.05% (w/v) (Murayama et al. 2007). In the sPMCA 0.05% (w/v) and 0.005% (w/v) was not able to reduce the formation of protease resistant PrP in healthy spiked and substrate only reactions. The failure of digitonin to reduce protease resistant rPrP may indicate different mechanistic formation of protease resistant PrP from bacterial and brain derived sources. In addition reports have been published in which protease resistant mouse PrP^{C} formation was reduced with the addition of digitonin, but the effect was only observed with antibodies directed to the C-terminal of PrP, antibodies such as SAF84 could be used to assess if this observation holds true for recombinant protease resistant PrP (Fujihara et al., 2009). α-crystallin is a heat shock protein which is expressed in neuronal cells in response to stress and interacts with amyloids inhibiting

their formation, including those of Aβ and α-synuclein proteins (Dehle et al., 2010; Head and Corbin, 1994; Shammas et al., 2011; Waudby et al., 2010). Furthermore in sPMCA α-crystallin has demonstrated both promotion (at 0.1 μg/ml) and inhibition (at 10 μg/ml) of PrP^{Sc} misfolding when vCJD prions were amplified in human brain substrate, therefore a range of concentrations were included in sPMCA reactions, but no dilution prevented the formation of protease resistant PrP (Barria, 2014). Again this may be the result of different folding tendencies of recombinant compared to brain derived PrP, however in RT-QuIC 10 μg/ml prevented formation of protease resistant PrP from recombinant human protein (Barria, 2014).

While recombinant hamster PrP has not yet proved to be an effective universal substrate in sPMCA for scrapie isolates, very recent evidence demonstrates that recombinant bank vole PrP is a very promising candidate in the *in vitro* amplification technique real-time quaking induced conversion (RT-QuIC) (Orrú et al., 2015). Utilising the recombinant bank vole RT-QuIC has been able to amplify 29 different strains of prion PrP^{Sc}, 14 from human prion diseases which include sCJD, vCJD, genetic and iatrogenic CJD, GSS and FFI. The remaining 15 animal prion diseases that were detected included ovine classical and atypical scrapie, murine scrapie, hamster scrapie and TME strains, CWD from deer and elk and bovine classical and L-type BSE. Subsequently, H-type BSE has also been amplified using truncated bank vole substrate in RT-QuIC (Masujin et al. 2016;Orrú et al. 2015; Orrú et al. 2015a).

The results described above and the previous observations by other researchers implies that recombinant PrP from hamsters, at least in sPMCA amplification, has a tendency to form spontaneous protease resistant β-sheet fibrils. This leads to false positive results particularly from those reactions seeded with healthy brain homogenates from scrapie free animals. The non-specific amplification and generation of protease resistant rPrP could not be resolved by the addition of either digitonin or α-crystallin to the sPMCA reactions. Before a universal quantitative sPMCA methodology can be produced with recombinant hamster PrP as substrate this issue must be addressed. With recent advances in RT-QuIC a more likely candidate for a universal substrate for sPMCA is recombinant bank vole PrP. With the current limitations of rPrP substrate developing a tool able to measure scrapie infectivity using qPMCA could not be accomplished, the focus of the study was switched to identify if correlation could be found between qPMCA replication efficiency and the *in vivo* infectivity of BSE isolates.

3.3.2. qPMCA

The current standard for measuring prion infectivity is the use of rodent bioassays which produce reliable infection rates and incubation periods that are related to the prion content of the sample. Dilutions of infectious materials can be used to assess the infective titres of prion containing materials. While these bioassays are accurate and reliable they are limited by the large amount of animals

required to carry out the experiments, the length of time required to perform these experiments and the high costs. Therefore there has been much interest in developing rapid *in vitro* replication assays with much lower costs that can produce quantitative outputs. One such *in vitro* assay is qPMCA which has been used to assess the infectivity of prions from a variety of sources for different applications.

The estimation of the concertation of infectious PrP^{Sc} particles has important implications for studying prion diseases in environmental, agricultural and in medical settings. The use of an *in vitro* sPMCA assay to estimate the concentration of PrP^{Sc} was first applied to hamster adapted scrapie prions (Chen et al., 2010). Chen and co-workers amplified a dilution series of a prion sample over a number of sPMCA rounds. By scoring positive signals over a background threshold value they were able to correlate the PrP^{Sc} content of the seed to the round at which a sample was considered a positive reaction. Using this correlation to produce a standard curve allowed the estimation of PrP^{Sc} in the brain, spleen, blood and urine of scrapie infected hamsters. The PrP^{Sc} content of the brain was estimated to be $2.3x10^{-5}$ g/g, with far smaller concentrations estimated in the spleen ($2.0x10^{-11}$ g/g), buffy coat ($2.6x10^{-13}$ g/ml), plasma ($1.3x10^{-14}$ g/ml) and urine ($2.0x10^{-16}$ g/ml).

Quantification of the infective prion load by qPMCA has been used for a variety of applications, in medical diagnostics to assessing the effective reduction in PrP^{Sc} load following decontamination methods. In the UK around 1 in 2000 people are estimated to be asymptomatic carriers of vCJD as judged from vCJD PrP^{Sc} content in appendix. vCJD can be transmitted iatrogenically and PrP^{Sc} is present in many tissues and fluids, therefore monitoring the asymptomatic carriage in the population is important to assess disease spread and prevent iatrogenic vCJD cases. qPMCA has been used to detect and quantify PrP^{Sc} in the urine of patients suffering from vCJD, and the assay had good sensitivity and specificity, no cross seeding activity was detected in samples from patients suffering from related protein misfolding disorders (Moda et al., 2014). The Estimation of PrP^{Sc} content in the urine of vCJD patients was conducted by comparing test samples to dilution series of an internal amplification control. The control consisted of healthy urine spiked with vCJD PrP^{Sc} from a brain homogenate, using recombinant protein of a known concentration the PrP^{Sc} concentration of the homogenate was estimated. Therefore urine spiked with known amounts of this brain homogenate could be used as an internal calibration and could be used to estimate the concentration of PrP^{Sc} in test samples at each round.

PrP^{Sc} is an extremely stable protein which is further stabilised when bound to stainless steel (Zobeley et al., 1999), as a result PrP^{Sc} is resistant to many treatments designed to routinely decontaminate medical instruments such as autoclaving (Brown et al., 1990). Iatrogenic CJD infections can occur through blood transfusions and surgery making medical instruments a potential source of infection (Jones et al., 2009; Wadsworth et al., 2001). Decontamination techniques that can reduce the

infectivity are therefore of critical importance and qPMCA has been used to assess the effectiveness of these techniques (Pritzkow et al., 2011). Hamster adapted scrapie strain 263K was bound to steel wires to model PrP^{Sc} adherence and stabilisation on medical instruments. The 263K PrP^{Sc} was then degraded by application of a variety of different chemical formulations, 16 in total were tested. By using an internal reference set of brain homogenate dilutions to control for variation between qPMCA runs which wasn't treated with denaturation agents, the degradation of PrP^{Sc} was measured in terms of reduction of qPMCA seeding activity. The products of this qPMCA appeared to still be biologically active, causing replication of PrP^{Sc} in a cell based assay. The quantification of the reduced seeding ability was directly compared to reduction of infectivity in rodent bioassays, and the qPMCA displayed strong correlation with the bioassay results. The use of qPMCA to assess decontamination of prions on medical instruments has also been applied directly to vCJD (Boerner et al., 2013).

Prion contamination of the environment is also a large concern due to the disposal of BSE contaminated cattle carcasses in the BSE epidemic and the contamination through shedding of TSE infected animals. Scrapie PrP^{Sc} has been demonstrated in a range of fluids including the urine, faeces, milk and saliva of infected sheep which could be deposited in the environment (Maddison et al., 2010c, 2009; Thorne and Terry, 2008). Decontamination of the environment poses unique challenges, as many chemical treatments have detrimental consequences in the treated areas. Therefore enzymatic subtilisin has been trialled as a method of reducing prion infectivity of TME in different soil types, before amplification in qPMCA to assess in which soil types the enzymatic treatment had led to the greatest decrease in PrP^{Sc} levels (Samuel E. Saunders et al., 2011). The estimation of PrP^{Sc} content was undertaken using TME with a defined infectious titre as measured by bioassay. This isolate was diluted in a 10 fold dilution series and amplified in sPMCA, the number of positive replicates at each round correlated strongly with the infectious dose in bioassay. The replication efficiency of TME treated with subtilisin in different soil types was then compared to this replication efficiency of the reference series, which enabled estimation of the remaining infectious titre.

The appropriate treatment of cattle waste and by-products is essential to prevent further BSE epidemic outbreaks, and to ensure that no new incidences of vCJD as a result of BSE contaminated food-stuffs occur. The effectiveness of wet and dry heat treatment of BSE contaminated tissue homogenates and macerates has been measured by assessing the sPMCA seeding activity following treatment (Matsuura et al., 2013). The reduction in seeding activity of the PrP^{Sc} was expressed as the amount of material required to seed only 50% of sPMCA reactions (SD_{50}) which was directly compared to the LD_{50} values attained in bioassay and a strong correlation was found between the two measures. Furthermore comparison of qPMCA SD_{50} to bioassay LD_{50} has been used to estimate the effectiveness of yellow grease heat treatment in the production of meat and bone meal (MBM) (Yoshioka et al.,

2013). The BSE epidemic in cattle was in part caused by the changing of MBM manufacturing processes, and therefore the processes used in the production of MBM is of crucial importance in preventing further BSE epidemics. Using qPMCA the optimal heating times of MBM in yellow grease to reduce BSE PrP^{Sc} seeding activity was determined to be 180°C for 3 hours. The qPMCA data was carried out in parallel with transmission studies to bovine transgenic mice, and again there was a strong correlation between the *in vitro* and *in vivo* assays.

Additional modifications to sPMCA methods have been made to increase the sensitivity and specificity of the techniques which in turn has made it more applicable to quantitative output. One such modification is the inclusion of Teflon beads to sPMCA reactions, using this method qPMCA successfully amplified hamster adapted scrapie strains 263K and SSLOW and derived SD_{50} values for each strain isolate (Natallia Makarava et al., 2012). The researchers found that the SD_{50} values were larger than the bioassay ID_{50} values, they proposed the difference was due to the relatively simple nature of *in vitro* amplification compared to disease progression in animal bioassays in which many factors such as host clearance of PrP^{Sc} all contribute to the ID_{50}.

Quantification of PrP^{Sc} has also been demonstrated in another *in* vitro amplification assay, RT-QuIC. RT-QuIC was developed to measure the infectivity of the nasal lavage of TME infected hamsters and brain and CSF of 263K scrapie infected hamsters. In both cases of TME and scrapie infectivity good correlation was observed with bioassays in terms of the SD_{50} and LD_{50}. Furthermore RT-QuIC was applied to deer infected with CWD, and was used to estimate the relative tissue distribution of CWD PrP^{Sc} of infected animals (Henderson et al., 2015a). CWD has a high rate of horizontal transmission, and as a results the infectivity was measured in the saliva and urine of deer, while the prion load was much lower than those of the infected brain they were still deemed large enough to be a significant reservoir for disease transmission.

While qPMCA methods detailed above have demonstrated reproducible amplification and quantification of a reference sample and subsequent test samples in correlation with *in vivo* infectivity, none have used one isolate as a reference to quantify the replication efficiency of an independent isolate which is also validated by comparison to *in vivo* infectivity in bioassay. This is a crucial advancement if qPMCA methodology is to be applied as a diagnostic assay that can measure *in vitro* replication efficiency and make meaningful estimates about *in vivo* infectivity as a result. In order to test if this level of reproducibility could be achieved in the absence of a rPrP substrate capable of amplifying scrapie from different genotypes, BSE was instead used as the PrP^{Sc} source in combination with brain derived PrP^{C}. The reason for this is that many strains of scrapie exist, each with different amplification efficiencies which are in part dependent on the genotype of the brain derived ovine substrates. Classical BSE on the other hand is thought to be comprised of only a single strain, which

would therefore display the same amplification efficiencies, as such it is much more suitable to testing inter isolate variation in qPMCA. Therefore amplification conditions were established which would enable measurement of *in vitro* replication efficiency. These consisted of the use of ovine substrate from animals with AHQ/AHQ genotype for *PRNP* (at amino acid positions 134, 156 and 171). Using this substrate amplification of a standard curve of BSE SE1946/0002 was used as a reference to estimate the brain content used to seed sPMCA reactions. qPMCA estimates of 26.75±10.72 µg and 1.59±0.87 µg were made for reactions seeded with 40 and 1.6 µg respectively. However when the amplification conditions were applied to 4 other BSE isolates with different LD_{50} values as measured in mouse bioassays, correlation between *in vivo* and *in vitro* data was not observed.

In summary, *in vitro* prion replication assays have been widely applied to estimate the levels of amplifiable PrP^{Sc} in a variety of sample types and in at least 2 studies the levels of amplifiable PrPSc has correlated with the infectious titre as estimated in rodent bioassay (Henderson et al., 2015a; Natallia Makarava et al., 2012; Matsuura et al., 2013; Pritzkow et al., 2011; Samuel E Saunders et al., 2011; Yoshioka et al., 2013). The present study set out to measure the accuracy of the qPMCA technique by estimating the spike content of known qPMCA replicates. The qPMCA predicted the content of infected brain material in the qPMCA spikes with reasonable accuracy for SE1946/0002 BSE. Replicates spiked with 40 µg and 1.6 µg of BSE brain material were estimated to contain 26.75±10.72 µg and 1.59±0.87 µg. For application of this technique as a valid replacement for animal bioassays that measure prion infectivity several improvements must first be made. While this estimation of amplifiable PrP^{Sc} was accurate (within 40% and 1%, depending on spike content) for a single BSE isolate. However when the sPMCA replication efficiencies of 4 BSE isolates were compared no correlation was found with infective load in mouse bioassay. The recombinant substrate trialled was able to amplify scrapie strains from a range of different *PRNP* genotypes, but large amounts of false positive results were also described which made the substrate unsuitable for qPMCA application.

Chapter 4. *In vitro* amplification and characterisation of atypical BSE

4.1. Introduction

After the discovery BSE in cattle in 1985 the infective agent showed consistent molecular and pathological traits even during passage in other species (Foster et al., 1993; Lasmézas et al., 1996; Wells et al., 1987). However, more recently, routine surveillance detected an atypical case of BSE in a non-symptomatic animal which had PrP^{Sc} of a low molecular weight for BSE, and unusual structure and distribution of amyloid plaques in the brain of the infected individual (Casalone et al., 2004). Further surveillance led to the description of another atypical BSE, H-type BSE, with a higher molecular weight than classical BSE and was characterised by the reactivity with the antibody P4 that binds at the N-terminal region of PrP (Biacabe et al., 2004). Importantly, transmission models indicate that the atypical BSE prions may present a significant zoonotic threat (Béringue et al., 2008; Comoy et al., 2008; Kong et al., 2008).

There has been some success in applying *in vitro* amplification assays, predominantly the real time quaking induced conversion assay (RT-QuIC), to amplify and detect both L-type and H-type BSE and differentiate these from classical BSE isolates (Orrú et al. 2015; Orrú et al. 2015b; Masujin et al. 2008). Although RT-QuIC can detect the presence of amyloidal seeding agents via fluorescence it is unable to generate PrP^{Sc} conformers which can be fully characterised. While *in vitro* amplification of atypical BSE has been fairly successful using RT-QuIC, amplification of the atypicals using sPMCA has not been as successful. H-type BSE has never previously been reported to amplify in sPMCA and successful L-type BSE amplification has only very recently been reported and required the addition of polyanions and L-arginine ethylester to the reactions (Murayama et al., 2016). Therefore this study aimed to amplify atypical prions from both L-type and H-type BSE in sPMCA and characterise the resultant PrP^{Sc} molecules to ensure faithful propagation of the initial seed and to enable differentiation between atypical and classical BSE.

4.2. Results

4.2.1. Optimisation of *in vitro* amplification of atypical BSE

The first stage in finding optimal conditions for amplification of both H-type and L-type atypical BSE were to identify which substrates would be most permissible for both atypical BSEs. Therefore an isolate of H-type (SE2053/0089) and L-type (SE2053/0106) BSE were amplified in duplicate in a panel of different substrates; bovine and ovine AHQ/AHQ, ARR/ARR and VRQ/VRQ genotypes (figures 4.1A and 4.2A respectively).

4.2.1.1. Optimisation of H-type BSE in PMCA

A single faint H-type PrP^{Sc} triplet was identified when a H-type isolate was amplified in bovine substrate (figure 4.1A marked with *). As no other amplicons were detected in any of the ovine substrates, bovine PrP^{C} was deduced to be the most suitable substrate for amplification of H-type BSE. To assess if the amplification of the H-type in bovine substrate could be increased further by variation in the duration of the sonication period, 5 replicates of H-type was amplified with 40, 30 and 20 seconds sonication lengths (figure 4.1B). At 40 seconds faint triplets were observed in all of the replicates, while faint PrP^{Sc} was only detected in 3 and 2 replicates of the 30 and 20 second sonication times respectively. Teflon beads have previously been shown to increase the specificity and sensitivity of PMCA reactions, therefore PMCA reactions at each sonication period were conducted with the inclusion of 3 Teflon beads (Gonzalez-Montalban et al., 2011). However the Teflon beads failed to increase the amplification efficiency of H-type BSE, no PrP^{Sc} was observed in any replicates when Teflon beads were included (figure 4.1C). A summary of the sPMCA conditions used to amplify H-type BSE is displayed in table 4.1.

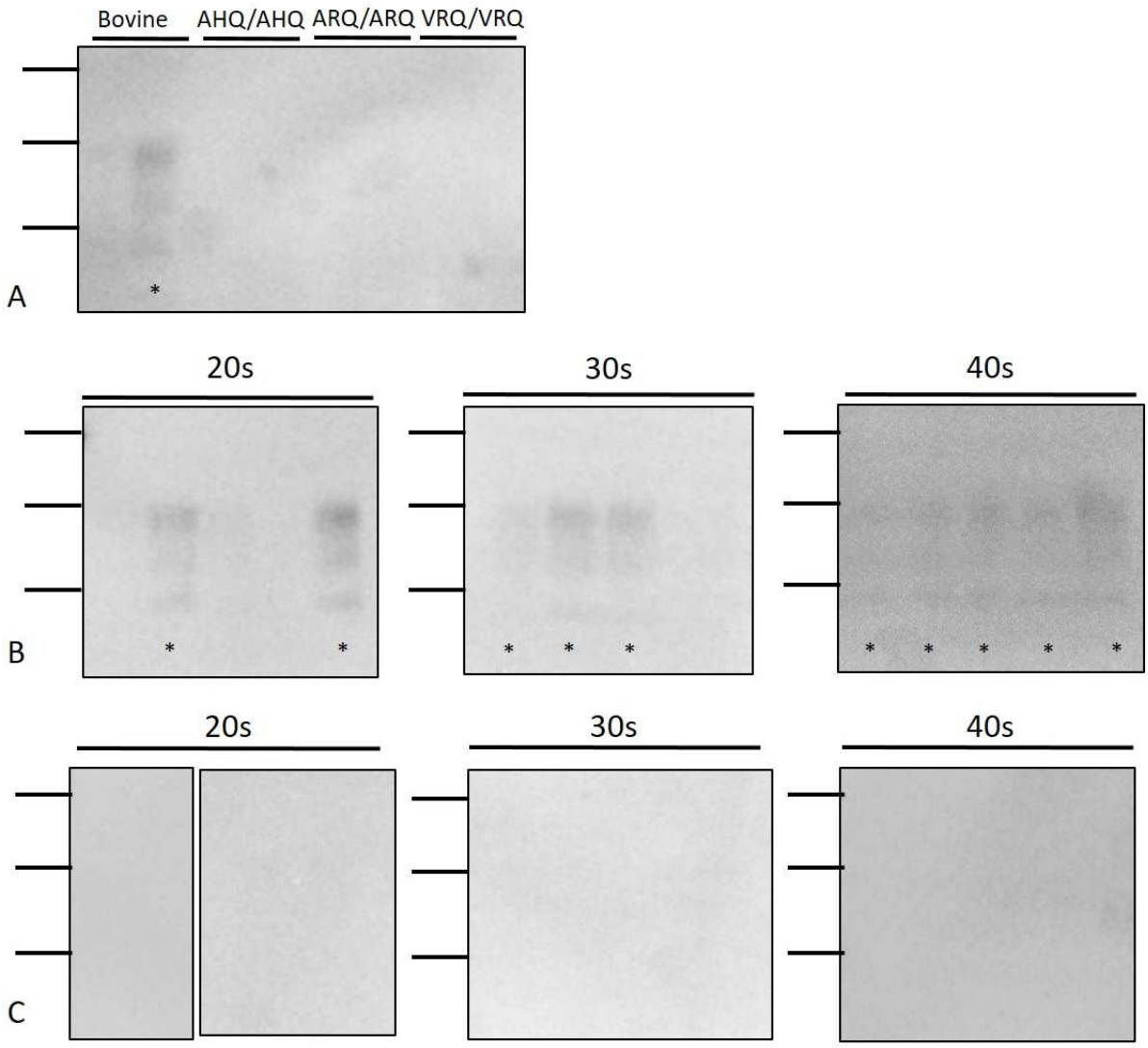


Figure 4.1. Optimisation of PMCA conditions for the amplification of H-type BSE. H-type BSE (SE2053/0089) was amplified for 5 rounds of PMCA before being digested with 50 µg/ml PK and electrophoresed by SDS-PAGE, the separated proteins were transferred to PVDF membrane and probed with SHa31 primary antibody. 40 30 and 20 kDa molecular markers are shown on the left of each blot. Faint amplicons are highlighted with *. **A**) H-type BSE was screened for amplification in different PrP^C substrates; bovine or AHQ/AHQ, ARR/ARR or VRQ/VRQ genotype ovine substrates as indicated. **B**) The length of the sonication period was reduced from the standard 40 seconds to 30 and 10 seconds in an attempt to improve the amplification efficiency of the PMCA in bovine substrate. **C**) Teflon beads were also included in PMCA reactions in addition to changing the sonication times displayed in panel **B**.

Table 4.1 Summary of sPMCA optimisation for the amplification of H-type BSE

Sonication time[a]	Substrate[b]	Teflon beads[c]	Proteinase K (μg/ml)[d]	Positive replicates[e]
40	bovine	-	50	1/2*
	AHQ/AHQ	-	50	0/2
	ARQ/ARQ	-	50	0/2
	VRQ/VRQ	-	50	0/2
40	bovine	-	50	5/5*
30		-	50	3/5*
20		-	50	2/5*
40	bovine	+	50	0/5
30		+	50	0/5
20		+	50	0/5
40	bovine	-	50	5/5*
		-	10	5/5
		-	5	5/5

a) The duration of the sonication period in the sPMCA (seconds)
b) The source of the 10% (w/v) brain homogenate used as substrate, either bovine or ovine AHQ/AHQ, ARQ/ARQ or VRQ/VRQ (amino acid positions 136, 154 and 171)
c) Presence (+) or absence (-) of 3 Teflon beads per sPMCA reaction
d) Concentration of PK used to digest sPMCA products after amplification
e) Number of positive reactions as visualised by reactivity with SHA31 antibody after SDS-PAGE Faint sPMCA products are indicated with *

4.2.1.2. Optimisation of L-Type BSE in PMCA

A very weak PrP^{Sc} signal was detected (highlighted with *) after L-type amplification in ovine substrate with genotype VRQ/VRQ at *PRNP* positions 136, 154 and 171 respectively (figure 4.2A). Replication of L-type PrP^{Sc} was not detected in PMCA reactions using bovine, or ovine AHQ/AHQ or ARQ/ARQ substrates. To improve the efficiency of the weak amplification of L-type BSE in VRQ/VRQ substrate, 5 replicates at 30 and 20 second sonication periods in PMCA were carried out in addition to the standard 40 seconds, however PrP^{Sc} was not found under either of the sonication conditions or the repeat of the 40s sonication condition (figure 4.2B). Teflon beads were then included in the sPMCA reactions in an attempt to increase amplification efficiency in each sonication time (figure 4.2C). The introduction of Teflon beads greatly increased the efficiency of amplification with 30s sonication

period, with 6/10 replicates producing strong PrP^{Sc} triplet signals. A single strong signal was also observed with 20 seconds sonication while the 40 seconds seemed to give a weak signal in one reaction. A summary of the conditions that were used to find the optimal sPMCA conditions for the amplification of L-type BSE is displayed in table 4.2.

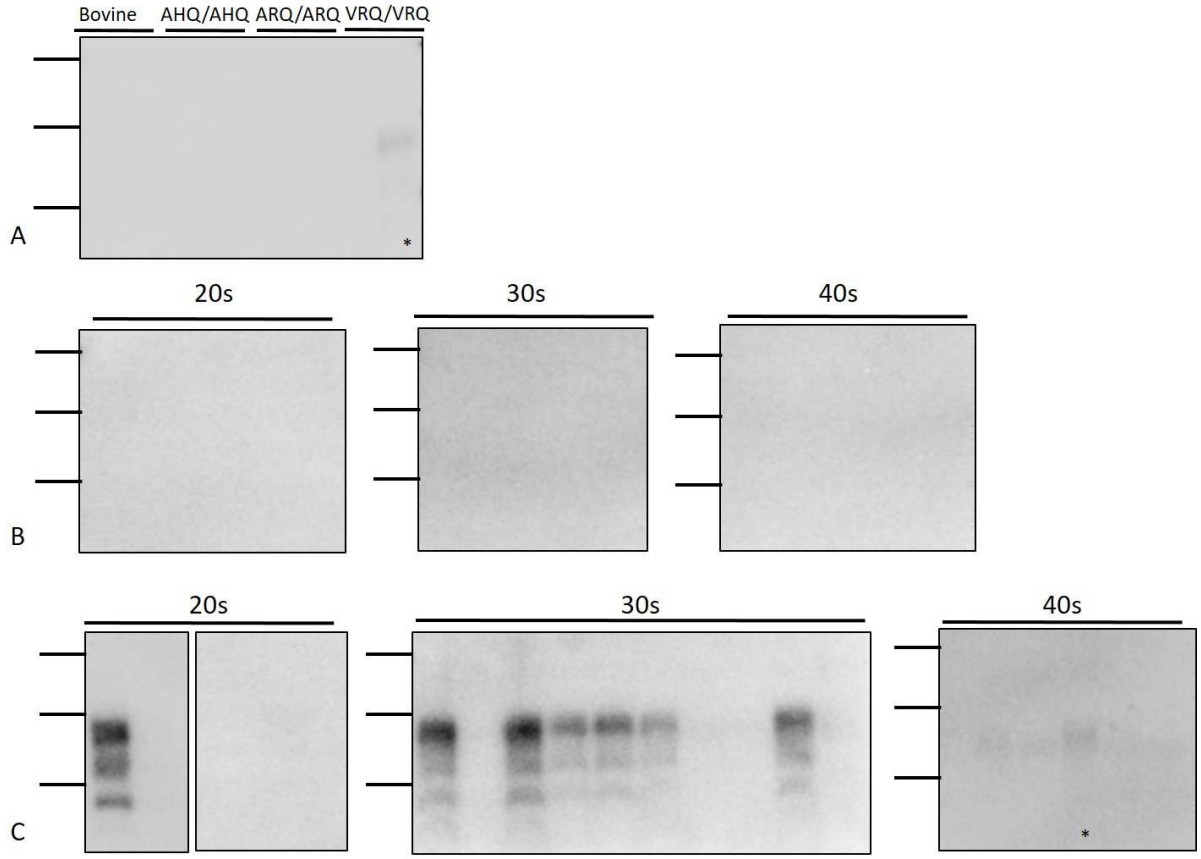


Figure 4.2. Optimisation of PMCA conditions for amplification of L-type BSE. L-type BSE (SE2053/0106) was amplified for 5 rounds of PMCA before being digested with 50 µg/ml PK and electrophoresed by SDS-PAGE, the separated proteins were transferred to PVDF membrane and probed with SHa31 primary antibody. 40 30 and 20 kDa molecular markers are shown on the left of each blot. Faint amplicons are highlighted with *. **A**) L-type BSE was screened for amplification in different PrP^{C} substrates; bovine, or AHQ/AHQ, ARR/ARR or VRQ/VRQ genotype ovine substrates as indicated. **B**) The length of the sonication period was reduced from the standard 40 seconds to 30 and 20 seconds in an attempt to improve the amplification efficiency of the PMCA in VRQ/VRQ substrates. **C**) Teflon beads were also included in PMCA reaction in addition to changing the sonication times displayed in panel **B**.

Table 4.2 Summary of sPMCA optimisation for the amplification of L-type BSE

Sonication time	Substrate	Teflon beads	Proteinase K (µg/ml)	Positive replicates
40	bovine	-	50	0/2
	AHQ/AHQ	-	50	0/2
	ARQ/ARQ	-	50	0/2
	VRQ/VRQ	-	50	1/2*
40	VRQ/VRQ	-	50	0/5
30		-	50	0/5
20		-	50	0/5
40	VRQ/VRQ	+	50	1/5*
30		+	50	6/10
20		+	50	1/5
30	VRQ/VRQ	+	50	2/5
		+	5	2/5

a) The duration of the sonication period in the sPMCA (seconds)
b) The source of the 10% (w/v) brain homogenate used as substrate, either bovine or ovine AHQ/AHQ, ARQ/ARQ or VRQ/VRQ (amino acid positions 136, 154 and 171)
c) Presence (+) or absence (-) of 3 Teflon beads per sPMCA reaction
d) Concentration of PK used to digest sPMCA products after amplification
e) Number of positive reactions as visualised by reactivity with SHA31 antibody after SDS-PAGE

4.2.1.3. Identifying relatively protease-sensitive PrP^{Sc} PMCA products

Amplification of H-type BSE generated very weak signals when digested with proteinase K at 50 µg/ml, and changes to the sonication time and addition of Teflon beads to the sPMCA reaction did not increase the strength of the signals, in contrast changes seemed to reduce amplification efficiency (figure 4.1A and 4.1B). As 5/5 replicates in PMCA with 40 seconds sonication time produced very weak signals these same replicates were digested with 10 and 5 µg/ml PK to ensure that the strength of the signal was not the result of efficient amplification of PrP^{Sc} with relatively low PK-resistance compared to the brain H-type PrP^{Sc} (figure 4.3A). Indeed, PrP^{Sc} triplets were observed producing strong western

blot signals in all 5 of the replicates when digested with lower PK concentrations indicating that the H-type sPMCA products were more PK sensitive than the H-type seed. The optimal conditions for amplification of H-type was then designated as 5 rounds of sPMCA in bovine substrate with 40 seconds of sonication between incubation stages and sPMCA products digested with 10 µg/ml PK.

The amplification efficiency of the L-type sPMCA using Teflon beads, VRQ ovine substrate and 30 seconds sonication was around 60%. The finding of PK sensitive H-type PrP^{Sc} raised the possibility that the negative L-type sPMCA reactions might also contain relatively PK sensitive products. Therefore 5 reactions (2 positive and 3 negative) initially digested with 50 µg/ml PK were treated with 5 µg/ml PK, however PrP^{Sc} was still not detected in the negative L-type BSE sPMCA products. Partially digested PrP^{C} was detected in the negative sPMCA reactions and this was also detected in the sPMCA reactions which contained no L-type spike, this has a molecular weight which lies between the mono-glycosylated and di-glycosylated PrP^{Sc} bands and has previously been described (Owen et al., 2007). In summary, the optimal conditions for the amplification of L-type PrP^{Sc} consisted of 5 rounds of sPMCA in VRQ ovine substrate with 30 seconds of sonication and the addition of 3 Teflon beads per sPMCA reaction before digestion with 50 µg/ml PK.

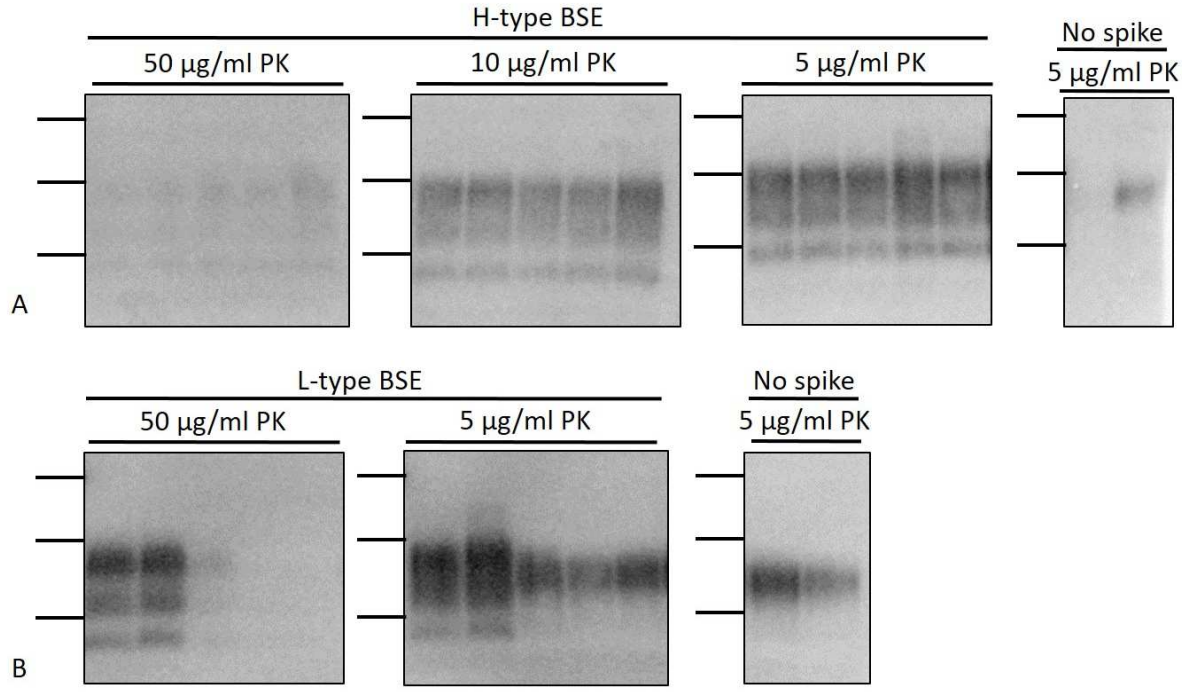


Figure 4.3. Identification of protease sensitive PrP^{Sc} in sPMCA products. A) H-type BSE sPMCA products amplified for 5 rounds in bovine substrate with a sonication period of 40s, then digested with 50, 10 and 5 µg/ml PK before being resolved on SDS-PAGE gels, transferred to PVDF membrane and probed with SHa31 antibody. **B**) L-type BSE was amplified for 5 round in ovine VRQ/VRQ substrate with 30 seconds sonication time, then digested with either 50 or 5 µg/ml PK, resolved on SDS-PAGE gels, transferred to blotting membrane and probed with SHa31 primary antibody. sPMCA

reactions containing only PrP^C substrate were amplified under H-L-type and L-type BSE sPMCA conditions and digested with 5 µg/ml PK prior to western blotting (indicated in **A** and **B**). Molecular markers of 40, 30 and 20 kDa are indicated on the left of each blot.

4.2.2. Limit of detection

After finding sPMCA conditions that could efficiently amplify both H-type and L-type BSE the limit of detection for the amplification of each set of conditions, and the ability of the sPMCA to amplify other classical and atypical BSE were determined.

Two isolates of H-type BSE (SE2053/0089 and SE1868/0011) and classical BSE (BP-1 and SE1762/0013) were amplified in H-type optimal sPMCA (bovine substrate, 40 second sonication, 10 µg/ml PK) containing between 1 mg and 1 pg of brain material of each isolate (figure 4.4). The sPMCA could detected between 1 ng and 1 pg of H-type BSE (summary in table 4.3). In an analogous experiment, classical BSE PrP^{Sc} was observed in reactions seeded with 1 and 10 ng of brain material. Faint amplification was also noted in one replicate out of 6 seeded with L-type BSE and amplified under these conditions containing 1mg of brain tissue. Ran alongside these amplifications were 20 negative control substrate only reactions, all of which produced only undigested PrP^C after digestion with 10 µg/ml PK (data not shown)

L-type BSE (SE2053/0106) was also subjected to sPMCA in ovine VRQ/VRQ substrate which had 30 second sonication time with the addition of three Teflon beads to find the limit of detection of this assay (figure 4.4). L-type BSE was detected only at the highest concentration of brain tissue in the seed, 10 µg, which only seeded PrP^{Sc} in 50% of the reactions. This data indicates that this sPMCA assay is on the very limit of detection at this concentration of spike. In addition, classical BSE was also amplified under the same conditions when analysing 1000 µg of brain spike (3/3 reactions). The data indicates that the assay can amplify both classical and L-type BSE prions.

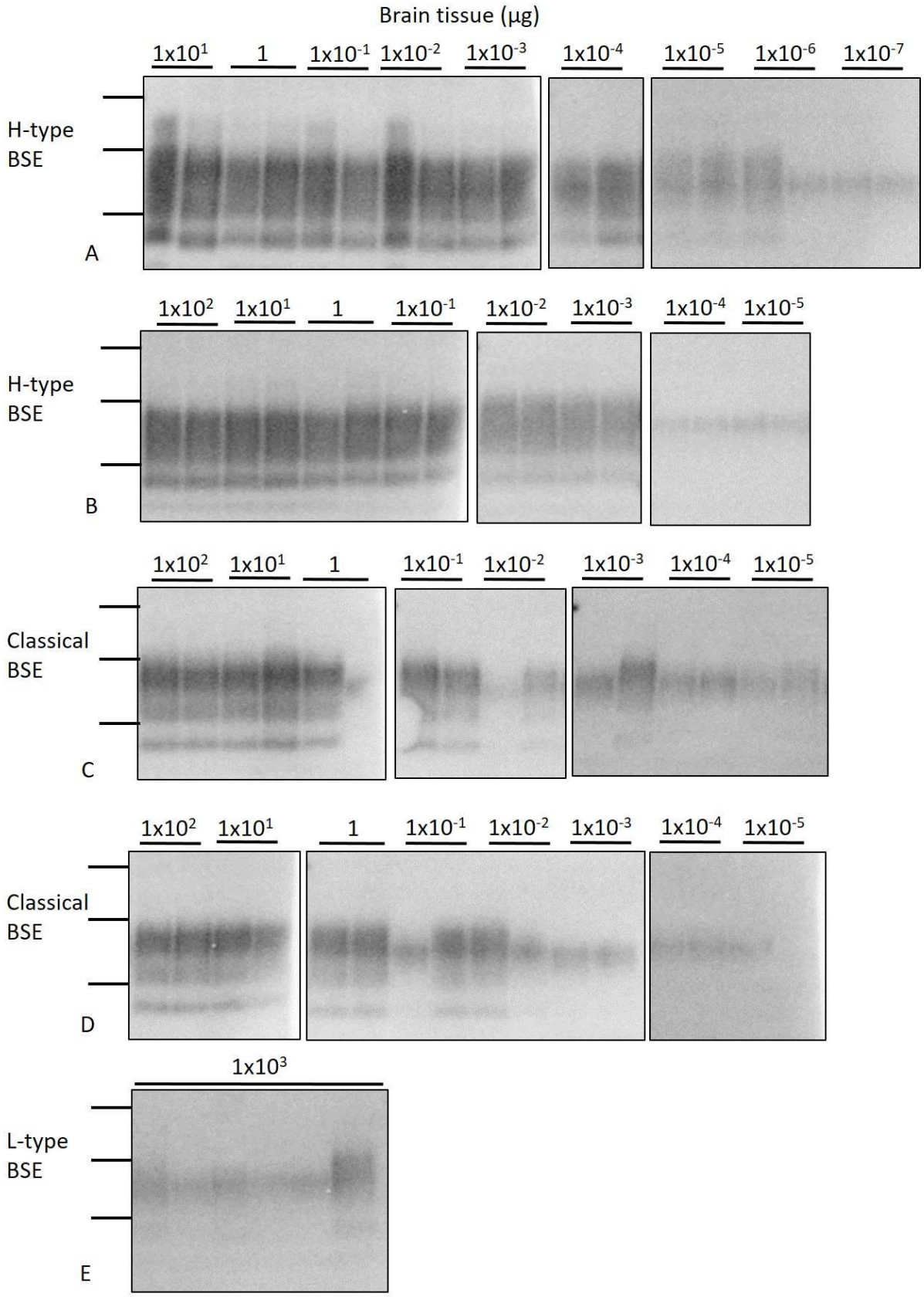


Figure 4.4. Limit of detection using sPMCA conditions to amplify H-type. A dilution series containing between 100 and $1x10^{-7}$ µg of brain tissue (as indicated) of H-type isolates SE2053/0089 (**A**), SE1868/0011 (**B**), classical BSE isolates BP-1 (**C**) and SE1762/0013 (**D**) and L-type BSE

SE2053/0106 (**E**), were amplified in 5 rounds of sPMCA ion bovine substrate with 40s sonication between incubation periods and were digested with 10 µg/ml PK before western blotting.

Table 4.3 Summary of limit of detection using H-type BSE sPMCA parameters

Brain material[a] (µg)	H-type BSE[b]		Classical BSE[c]	
	SE2053/0089	SE1868/0089	BP-1	SE1762/0013
$1x10^{2}$	N/A	2/2	2/2	2/2
$1x10^{1}$	2/2	2/2	2/2	2/2
1	2/2	2/2	1/2	2/2
$1x10^{-1}$	2/2	2/2	2/2	1/2
$1x10^{-2}$	2/2	2/2	1/2	1/2
$1x10^{-3}$	2/2	2/2	1/2	0/2
$1x10^{-4}$	2/2	0/2	0/2	0/2
$1x10^{-5}$	2/2	0/2	0/2	0/2
$1x10^{-6}$	1/2	N/A	N/A	N/A
$1x10^{-7}$	0/2	N/A	N/A	N/A

a) The wet weight of brain material used to seed sPMCA reactions
b) The number of positive H-BSE replicates after sPMCA amplification
c) The number of positive classical BSE replicates after sPMCA amplification

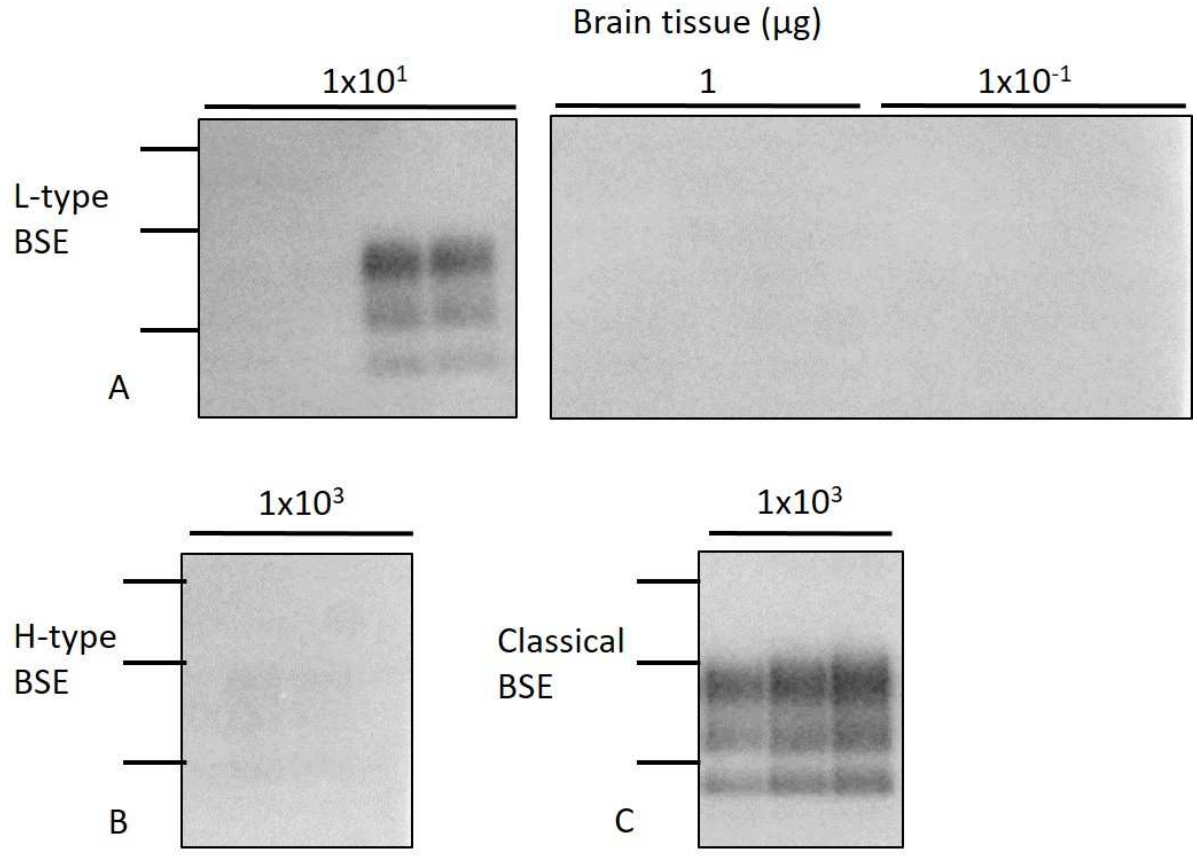


Figure 4.5. Limit of detection of L-type BSE in sPMCA. (**A**) 10, 1 and 0.1 µg of brain tissue of L-type BSE isolate (SE5023/0106), or (**B**) 1000 µg of H-type BSE SE1868/0011 or (**C**) classical BSE SE1762/0013 of brain material was diluted in ovine VRQ/VRQ substrate and amplified in 5 rounds of sPMCA with 30 seconds sonication times and 3 Teflon beads per reaction. The sPMCA products were then digested with 50 µg/ml PK, electrophoresed by SDS-PAGE and separated proteins transferred to PVDF membrane and probed with SHa31 primary antibody.

4.2.3. Biochemical characterisation of atypical and classical BSE PrP^{Sc} amplified in sPMCA

4.2.3.1. Conformational stability of sPMCA generated PrP^{Sc}

Treatment of PrP^{Sc} with guanidine hydrochloride (Gdn-HCl) unfolds the prion protein conformers and increases susceptibility to protease degradation. Different conformers of PrP^{Sc} have distinct sensitivities to Gdn-HCl treament, therefore different PrP^{Sc} molecules can be distinguished using this principle in conformational stability assays (CSA), which has already been applied to various scrapie and BSE isolates, including the differentiation of L-type and classical BSE isolated (Di Bari et al., 2013; Masujin et al., 2008; Peretz et al., 2001; Pirisinu et al., 2010; Vrentas et al., 2013). Here, it was assessed whether CSA could distinguish classical, L-type and H-type BSE both before and after amplification by sPMCA.

Two 10% (w/v) brain homogenates of classical BSE, SE1762/0013 and BP-1 with the addition of sPMCA products spiked with SE1762/0013 (amplified under H-type optimised sPMCA conditions) were subjected to denaturation with concentrations of Gdn-HCl at between 0.5 and 4.0 M before digestion with 50 µg/ml PK (figure 4.6). H-type BSE 10% (w/v) brain homogenate SE1868/0011 and SE2056/0089 spiked sPMCA products (amplified using conditions optimal for H-type PMCA amplification) in addition to L-type BSE 10% (w/v) brain homogenates SE1868/0008 and SE2053/0106 spike sPMCA products (amplified using conditions optimal for L-type PMCA amplification) were also treated with Gdn-HCl (figures 4.7 and 4.8 respectively). After Gdn-HCl treatment, H-type BSE was digested with 10 µg/ml PK and L-type BSE with 50 µg/ml PK before western blotting and the signal from the western blots measured in ImageJ software and expressed as a percentage signal of the lowest Gdn-HCl treatment signal (0.5 M). The percentage of the 0.5 M signal for each subsequent Gdn-HCl concentration was then plotted against the concentration in GraphPad Prism and lines of best fit for each curve added using sigmoidal dose response curve functions. These were used to generate the Gdn_{50} values (concentration of Gdn-HCl responsible for 50% reduction in signal compared to 0.5 M treatment) as a measure of conformational stability of PrP^{Sc} (table 4.4).

From the plots of Gdn-HCl concentration against the western blot signal of the PrP^{Sc} triplet it was clear that the PrP^{Sc} from the sPMCA products were more susceptible to Gdn-HCl degradation for all samples analysed: the classical, H-type and L-type BSE products, a finding that also supported by the Gdn_{50} values (figure 4.9, table 4.4). Statistical analysis of the Gdn_{50} values in a one-way ANOVA (using GraphPad Prism statistical package) found a significant difference (P=0.0005) between the means of all the Gdn_{50} values, however using the same one-way ANOVA test but splitting the Gdn_{50} values into two groups, sPMCA products and 10% (w/v) brain homogenates, the differences within these groups was not found to be significant (P values of 0.1345 and 0.112 respectively). Furthermore two-tailed unpaired T-tests (using GraphPad Prism statistical package) were applied to the Gdn_{50} values of sPMCA products seeded with classical, H-type and L-type BSE but neither of the three tests found the difference between the Gdn_{50} means significant. The results of the split one-way ANOVA and t-tests imply that the difference detected in the initial one-way ANOVA was related to the increased sensitivity to denaturation of the sPMCA products compared to the brain homogenates, rather than a difference between the different BSE isolates. The same relationship was found when analysing the percentage denaturation at 3.0 M Gdn-HCl, a method which has previously been applied to CSAs (Pirisinu et al., 2011). At this concentration of Gdn-HCl there was a significant difference in denaturation when all groups were tested (P=0.0011), however when split into sPMCA amplified H-type, L-type and classical BSE samples a significant difference was not observed (P=0.3166) although a difference between 10% (w/v) brain homogenates was significant (P=0.0001) (all analysed in one-way ANOVA).

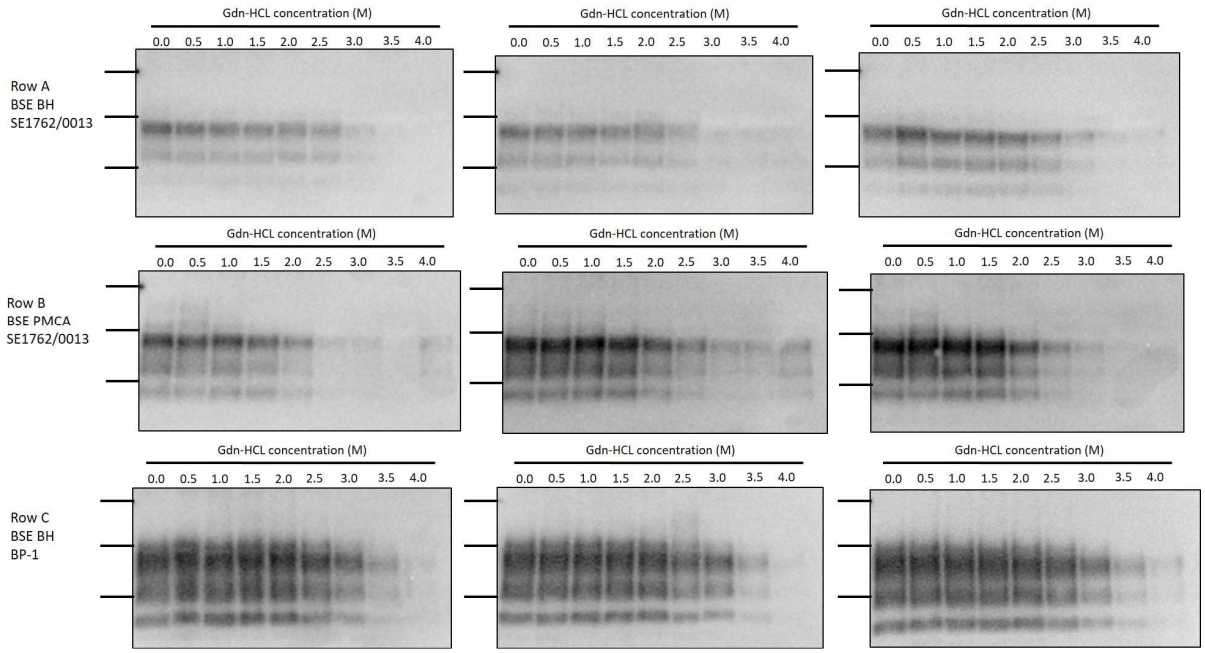


Figure 4.6. Classical BSE conformational stability assay western blots. Two Bovine BSE 10% (w/v) brain homogenates SE1762/0013 (row **A**) and BP-1 (row **C**), SE1762/0013 PMCA products after

5 rounds of amplification in VRQ/VRQ substrate (row **B**) were subjected to denaturation with Gdn-HCl (at the concentrations indicated). Samples were then digested with PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SHa31 antibody. 40, 30 and 20 kDa markers are shown to the left of each blot.

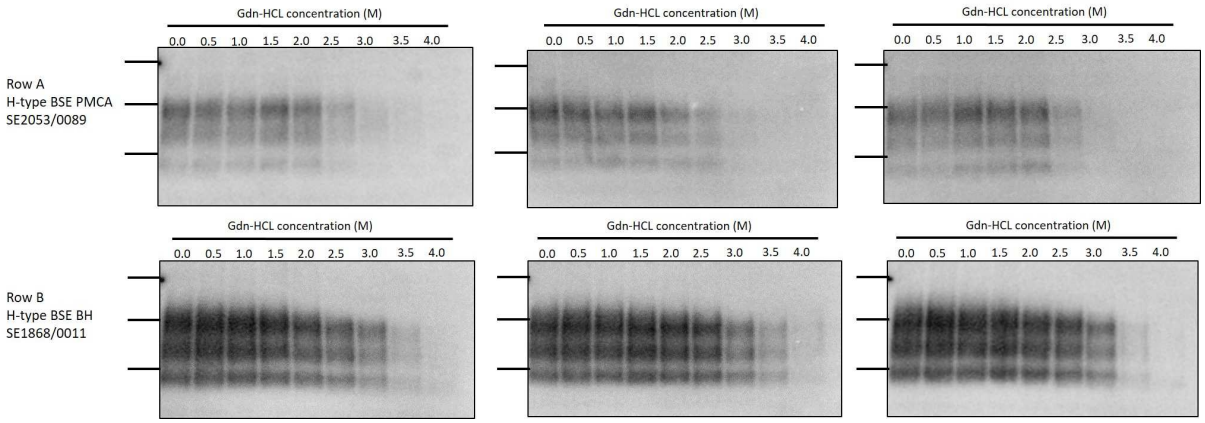


Figure 4.7. H-type BSE conformational stability assay western blots. An isolate of H-type BSE (SE2053/0089) amplified in sPMCA (row **A**) and a 10% (w/v) H-type BSE brain homogenate (SE1868/0011) (row **B**) were denatured by exposure to Gdn-HCl. Samples were then treated with various concentrations of Gdn-HCl prior to being digested with PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SHa31 antibody. 40, 30 and 20 kDa markers are shown to the left of each blot.

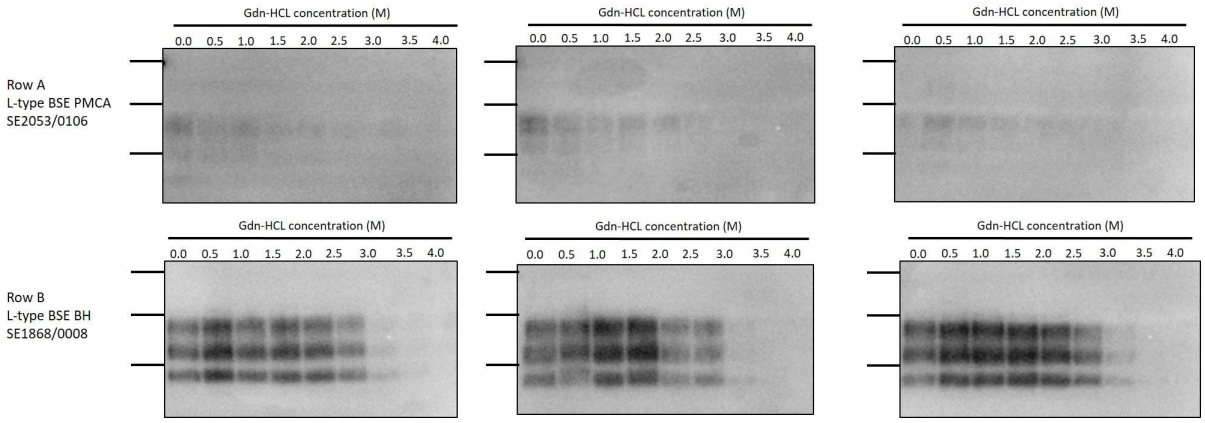
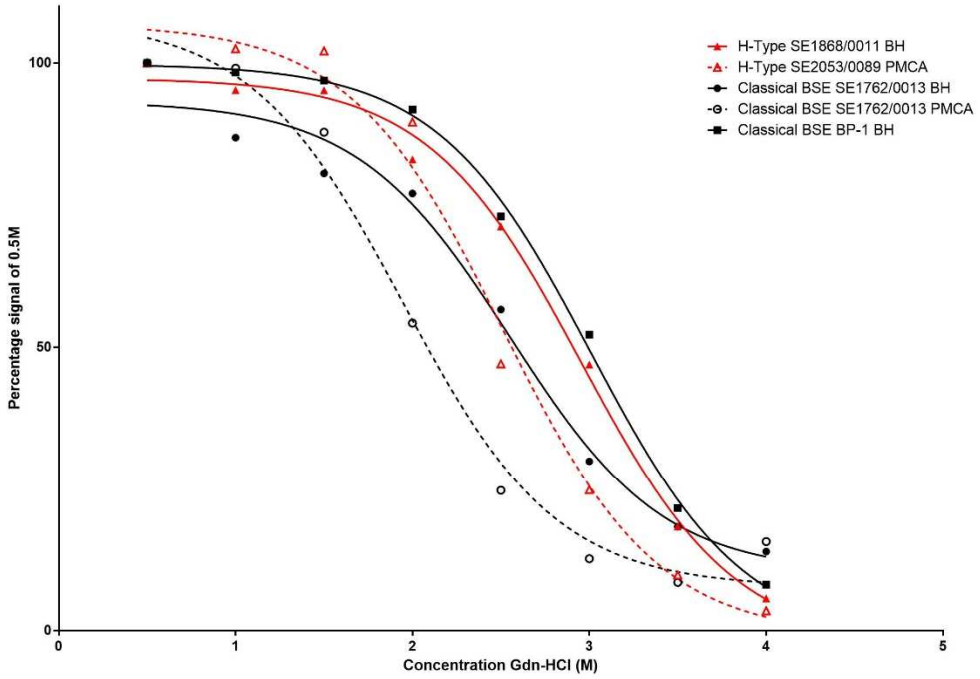
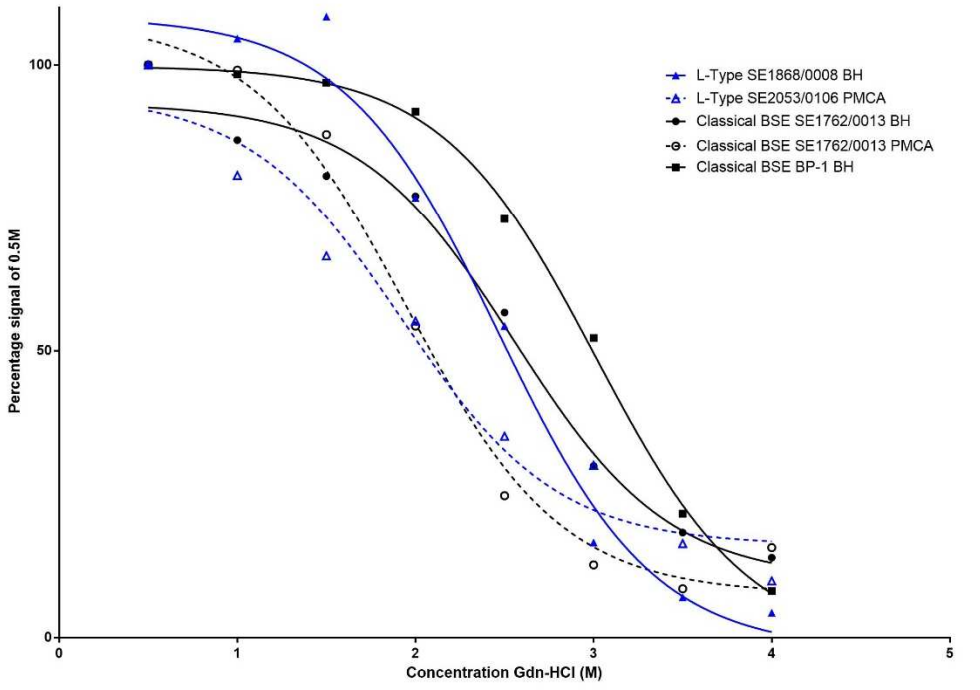


Figure 4.8. L-type BSE conformational stability assay western blots. An isolate of L-type BSE (SE2053/0106) amplified in sPMCA (row **A**) and a 10% (w/v) L-type BSE brain homogenate (SE1868/0008) (row **B**) were denatured by exposure to Gdn-HCl. Samples were then treated with various concentrations of Gdn-HCl prior to being digested with PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SHa31 antibody. 40, 30 and 20 kDa markers are shown to the left of each blot.



A



B

Figure 4.9. Conformational stability of H-type and L-type BSE as a function of signal at 0.5M guanidine hydrochloride (Gdn-HCl). The concentration of Gdn-HCl was plotted against the percentage loss of signal as a measure of denaturation by incubation with Gdn-HCl, for classical BSE controls and H-type BSE (**A**) or L-type BSE (**B**) 10% (w/v) brain homogenates and sPMCA products. Incubation with 0.5 M Gdn-HCl was treated as the baseline for denaturation and PrP^{Sc} signals produced using other concentrations of Gdn-HCl was expressed as a percentage of the 0.5 M signal.

The lines of best fit were plotted using the sigmoidal dose-response curve function in GraphPad Prism from the three replicates of the denaturation series.

Table 4.4 Conformation stability represented as Gdn_{50} values for classical, H-type and L-type BSE

Sample[a]	Gdn_{50}[b]	
	Brain homogenate	PMCA product
H-type BSE	2.94 ± 0.167	2.46 ± 0.225
L-Type BSE	2.47 ± 0.090	1.76 ± 0.461
Classical BSE SE1762/0013	2.55 ± 0.181	1.95 ± 0.091
Classical BSE BP-1	3.01 ± 0.120	-

a) The isolate of BSE the conformational stability assay was applied to
b) 50% reduction in signal due to Gdn-HCl treatment of either 10% (w/v) brain homogenates or sPMCA products after 5 rounds of amplification. H-type brain homogenate – SE1868/0011 and sPMCA spike – SE2053/0089. L-type brain homogenate – SE1868/0008 and sPMCA spike – SE2053/0106.

4.2.3.2. Differentiating H-type and classical BSE sPMCA products by protease susceptibility and cleavage sites

To apply sPMCA as a detection and screening method that could identify very low levels of BSE and importantly also differentiate H-type BSE from classical BSE, further characterisation and discrimination of the PrP^{Sc} in the sPMCA products is necessary.

4.2.3.2.1. Estimation of molecular weights

The molecular weights of PrP^{Sc} have previously been used differentiate between classical and H-type BSE (Biacabe et al., 2004). The molecular weights of the sPMCA products were analysed to confirm if the sPMCA products retain the associated molecular weights of each BSE isolates and this included at high and low spikes produced for the limit of detection of H-type BSE in the optimised sPMCA conditions. The highest concentration of brain tissue used to seed sPMCA reactions was 100 µg for each isolate, the lowest concentrations of spikes varied. For H-BSE spikes this was 1 ng brain material while the lowest spike of both classical BSE isolates were 100 ng of brain tissue per sPMCA reaction.
The migration distance of H-type and classical BSE un-glycosylated PrP^{Sc} that were detected using SHa31 primary antibody (figure 4.10A) were compared to the linear regression curve of the migration of the 60, 50, 40, 30 and 20 kDa molecular weight markers to estimate the molecular weights of the un-glycosylated PrP^{Sc} of each sPMCA product of H-type and classical BSE (figure 4.10B). The difference

in molecular weight was conserved for both H-type BSE isolates at high and low spikes with a mean of 16.72 kDa. The mean molecular weight of the classical BSE un-glycosylated band was also conserved at high and low spikes for both isolates, with a mean of 15.43 kDa. This difference between the two un-glycosylated bands was deemed significant by a two-tailed unpaired t-test (P<0.0001).

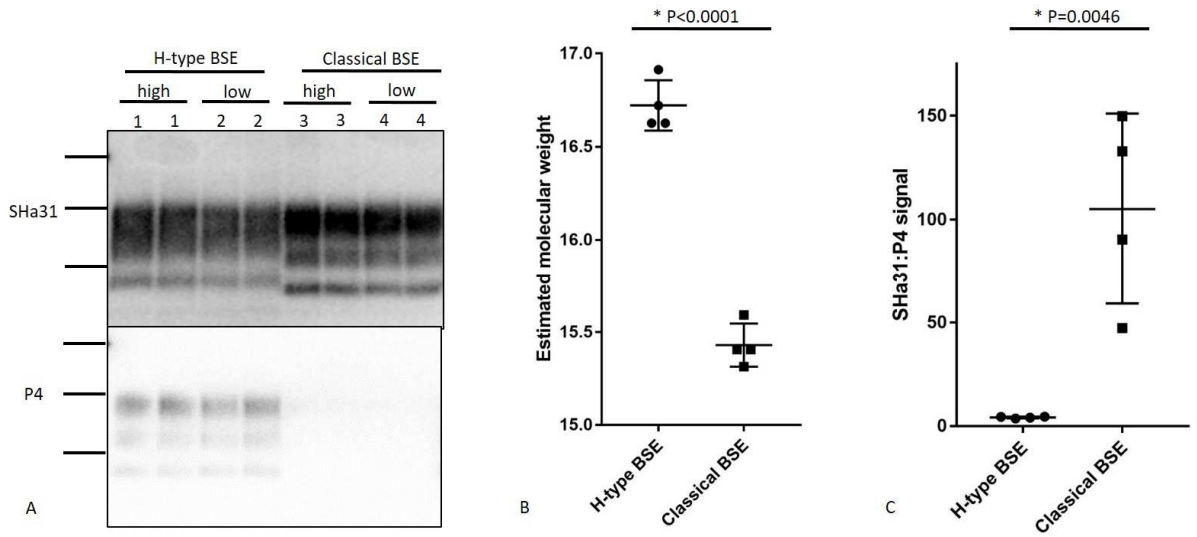


Figure 4.10. Estimation of molecular weight and P4 reactivity using of H-type and classical BSE. **A**) H-type BSE and classical BSE isolates were used to seed sPMCA reactions and were amplified for 5 rounds in sPMCA using H-type optimised conditions including digestion with 10 µg/ml PK. H-type BSE spikes were isolates SE2053/0089 (**1**) and SE1868/0089 (**2**) and classical BSE spikes BP-1 (**3**) and SE1762/0013 (**4**). The sPMCA products were generated using seeds at both a high or low concentration to assess if the characteristics of PrP^{Sc} were consistent at different levels of spike. The high spike was 100 µg of brain tissue per sPMCA reaction for each H-type and classical BSE. Low spikes consisted of 1 ng for H-types (**1** and **2**) and 100 ng for classical BSE (**3** and **4**). After PK digestion the proteins were separated in SDS-PAGE and transferred to PVDF membranes before probing with either SHa31 or P4 primary antibodies as indicated. Molecular markers of 40, 30 and 20 kDa are indicated to the left of each blot. **B**) The molecular weights of the un-glycosylated bands of each H-type and classical BSE replicate detected using the SHa31 antibody were analysed by comparison to the linear regression of the standard molecular weight markers at 60, 50, 40, 30 and 20 kDa. The difference in the means of the classical and atypical BSE was compared using a two-tailed unpaired t-test, in GraphPad Prism, which gave a P value <0.0001. **C**) The ratio of signal produced when probed with SHa31 and P4 antibodies for H-type and classical BSE (**A**) was measured in ImageJ expressed as a function of an internal blotting control. The difference in the means of the H-type and classical BSE was significant when measured using a two-tailed unpaired t-test with a P value of 0.0046.

4.2.3.2.2. H-type and classical BSE reactivity with secondary antibody P4.

H-type BSE, unlike classical BSE, reacts with primary antibody P4 and this difference can be used to identify H-type BSE PrP^{Sc}. PrP^{Sc} triplets were detected in H-type and classical BSE sPMCA products generated from high and low spikes that were probed with SHa31, however for PrP^{Sc} probed with P4 antibody, only H-type PrP^{Sc} was detected (figure 4.10A). The Sha31 and P4 signal ratio was expressed as a function of an internal blotting control and plotted in GraphPad Prism (figure 4.10C). The ratio of SHa31 signal to P4 for H-type BSE had a mean of 4.2, but the ratio for classical BSE was much higher with a mean of 105.1. The loss of P4 signal in classical BSE was significantly different when analysed by a two-tailed unpaired t-test (P=0.0046).

4.2.3.2.3. H-type BSE sensitivity to protease digestion

H-type BSE has been demonstrated to have different susceptibility to classical BSE in mild and stringent digestion conditions (Jacobs et al. 2007). During the optimisation of the sPMCA conditions the products of the amplification of H-type BSE spikes were found to have greater sensitivity to protease degradation than L-type or classical BSE (figure 4.3 and data not shown). The sPMCA products that were produced with high and low spikes of H-type and classical BSE were digested with both 10 and 50 µg/ml PK prior to western blotting and probing with SHa31 antibody (figure 4.10A and 4.10B). H-type isolates SE2053/0089 (figure 4.10A) and SE1868/0011 (figure 4.10B) at both high and low spikes were repeatedly detectable only after digestion with 10 µg/ml PK, whereas PrP^{Sc} triplets from classical BSE isolates BP-1 (figure 4.10A) and SE1762/0013 (figure 4.10B) were repeatedly present after digestion with both 10 and 50 µg/ml PK. When the ratio of these signals were analysed the increased susceptibility of H-type BSE to PK was significantly different to the classical BSE (P=0.008) in a two-tailed unpaired t-test (figure 4.10C).

Research has also shown that this difference in susceptibility can be analysed by using mild and stringent digestion conditions (Jacobs et al. 2007). Adaptation of this assay here used ‘mild’ digestion conditions consisted of 10 µg/ml PK and pH 6.5 and ‘stringent’ digestion conditions of 100 µg/ml PK at pH 8.0. H-type BSE was again more susceptible to digestion in the more stringent conditions as described in figure 4.10A and 4.10B, as previously reported in the literature (figure 4.10D).

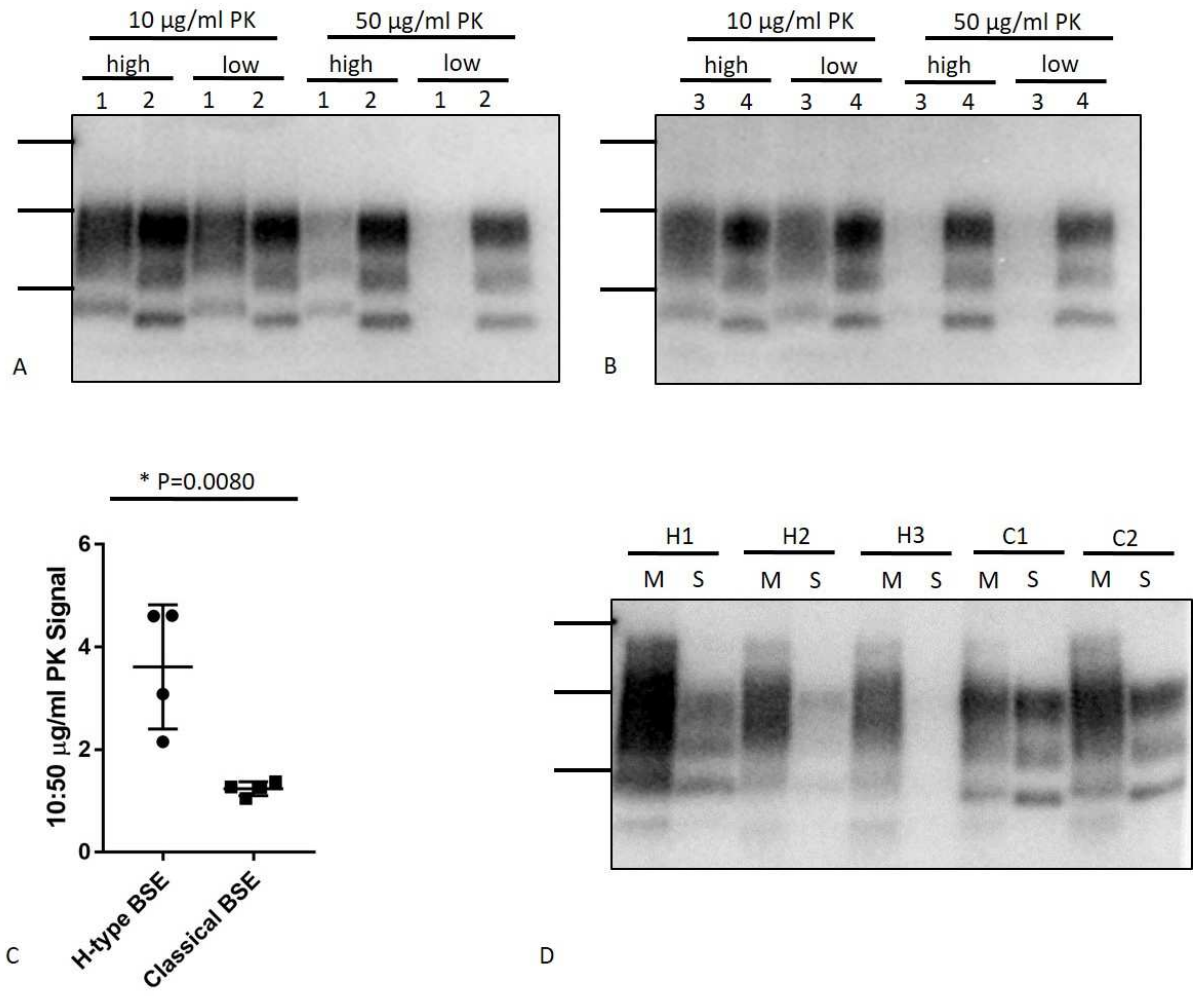


Figure 4.11. Protease susceptibility of classical and H-type BSE. A and **B**) H-type and classical BSE isolates were digested with either 10 or 50 µg/ml PK before western blotting and probing with SHa31 antibody. H-type SE2053/0089 (**1**) and classical BP-1 (**2**) are displayed in A and H-type SE1868/0011 (**3**) classical SE1762/0013 (**4**) isolates in B. The high spike consisted of 100 µg of brain tissue per sPMCA reaction for each H-type and classical BSE. Low spikes consisted of 1 ng for H-type BSE and 100 ng for classical BSE isolates. **C**) The ratio of signal observed between 10 and 50 µg/ml PK for the H-type and classical BSE isolates. Difference of means significant by two-tailed unpaired t-test (P=0.0080). **D**) Digestion of H-type and classical BSE isolate at mild (**M**) (10 µg/ml PK at 6.5 pH) and stringent (**S**) (50 µg/ml PK at 8.0 pH) conditions. H-type 10% (w/v) brain homogenate SE1868/0011 (**H1**) and H-type PMCA products SE2053/0089 (**H2**) and SE1868/0011 (**H3**). Classical BSE sPMCA products BP-1 (**C1**) and SE1762/0013 (**C2**). Molecular markers of 40, 30 and 20 kDa are indicated to the left of each blot.

4.3 Discussion

Atypical BSE in cattle tend to occur in older animals than with classical BSE infections. Usually atypical cases occur in individuals around 12 years of age yet onset for classical BSE is less than half that figure,

on average between 5 and 5.5 years (Arnold and Wilesmith, 2004; Brown et al., 2006; Wilesmith et al., 1988). In addition to the late onset of disease, atypical BSE infections are almost always asymptomatic and as a results are only detected in animals as part of active surveillance, these features are hallmarks of many spontaneous human prion diseases which may indicate that atypical diseases might also be spontaneous in origin (Seuberlich et al., 2010).

While there is no direct evidence of atypical BSE infecting humans through infected food-stuffs, the nature of prion infections would make the acquisition of such evidence extremely difficult to come by. However model infections for human susceptibility to L-type and H-type in humanised transgenic mice have confirmed that there is a possible zoonotic threat posed by these prions, which is supported by the transmission of L-BSE to a macaque (Béringue et al., 2008; Comoy et al., 2008; Kong et al., 2008). Each of these studies compared atypical BSEs to classical BSE, in each instance the infectivity of the atypical BSEs was greater than that of the classical BSE isolates, in the model systems used.

As a result of the asymptomatic carriage of atypical BSE, the potentially larger zoonotic potential over classical BSE and relatively low levels of atypical PrP^{Sc} deposition in tissues routinely tested for prions in cattle by conventional immunohistochemistry and biochemical tests; there has been a large interest in *in vitro* amplification methods to detect very low levels of atypical BSE PrP^{Sc} and differentiate it from classical BSE. To date the only published amplification of atypical BSE using sPMCA has been applied to L-type BSE in macaques. This study was published towards the end of this PhD study and reported sPMCA which utilised mouse PrP^{C} as substrate and several additives which included polyanions and L-arginine ethylester. The assay was highly sensitive, able to detect PrP^{Sc} in brain material in $1x10^{-11}$ dilutions. Furthermore, PrP^{Sc} was detected in asymptomatic carriage in the saliva, urine and CSF of infected individuals (Murayama et al., 2016). However, it was not determined whether the sPMCA assay could distinguish L-BSE from other BSE types.

An alternative *in vitro* amplification technique known as quaking induced conversion (QuIC) has been applied to amplify both H-type and L-type BSE. QuIC studies have been published which employ several truncated versions of recombinant hamster PrP^{C} and bank vole substrate to amplify L-type BSE and differentiate from classical BSE (Christina D Orrú et al., 2015; Christina D. Orrú et al., 2015). Using these substrates QuIC can detect atypical BSEs at dilutions between $1x10^{-6}$ and $1x10^{-7}$ for L-type and $1x10^{-8}$ and $1x10^{-9}$ for H-type which they find comparable to bioassays. In addition, QuIC assays have been used to differentiate between classical, H-type and L-type BSE via amplification in both bank vole and ovine ARR/ARR PrP^{C} although the sensitivity of this assay was at dilutions of brain material between 10^{-3} and 10^{-5} of clinically affected cattle (Masujin et al., 2016). Classical BSE isolates only amplify in the bank vole substrates, which also amplify H-type much more efficiently than L-type. In the ovine substrate classical BSE fails to amplify and L-type is more efficient than H-type, using the

two substrates this assay could be used to differentiate unknown BSE isolates, providing the each contained sufficient PrP^{Sc}.

In this study the efficient amplification of H-type BSE in sPMCA has been documented for the first time, as has amplification of L-type BSE in PMCA conditions which did not require additional chemical additives. The optimised sPMCA for H-type BSE was able to detect minute quantities of seed which related to between 1 ng of brain material; or 10^{-10} dilution of brain material. The sPMCA was able to differentiate between H-BSE and classical BSE by further biochemical analysis of the sPMCA products, and did not amplify L-type BSE efficiently. Differentiation of atypical and classical BSE sPMCA products by the conformational stability assay proved unsuccessful. A previous report stated that bovine BSE, and atypical BSEs are not readily denatured by Gdn-HCl and the Gdn_{50} value does not appear lower than ~3.0 M (Masujin et al., 2008; Vrentas et al., 2013). In this study classical bovine BSE and atypical BSE was more sensitive to Gdn-HCl with Gdn_{50} values between 2.55 and 3.01, but after amplification these dropped to below 2.0 M for classical BSE. Conformational stability of sPMCA products has not been widely reported before, and it was clearly demonstrated that the PrP^{Sc} sPMCA products for classical and atypical BSEs were more sensitive to denaturation than the original PrP^{Sc} in brain.

The sPMCA was not sensitive enough for L-type BSE (detecting only 10 ng of brain tissue) for practical application as a surveillance method or diagnostic test when *in vitro* assays already exist with similar sensitivities, therefore further strain typing methods were not carried out on L-type sPMCA products (Orrú et al. 2015a; Murayama et al. 2016). However, with the very high sensitivity of the sPMCA for H-type BSE and the amplification of classical BSE under the same conditions, attempts were made to differentiate these two PrP^{Sc} products. When using both high and low seeds of brain homogenate to generate sPMCA products, H-type PrP^{Sc} was readily differentiated from classical BSE based on the molecular weight of its unglycosylated PrP^{Sc} band, its reactivity with the N-terminal antibody P4, and its increased sensitivity to proteinase K treatment. It is possible that this increased sensitivity of H-type BSE sPMCA products to proteinase digestion, which is not apparent in the seed, is the reason other sPMCA methods have not been identified which amplify H-type BSE. Likewise, the success of RT-QuIC to produce effective H-type screening methods may lie in the fact that RT-QuIC does not detect PK-resistant PrP^{Sc} but the presence of amyloidal aggregates via fluorescence, and therefore does not require a stringent proteinase degradation step (Atarashi et al., 2011).

In summary, the study describes amplification of L-type BSE minus chemical sPMCA additives, and the efficient amplification of H-type BSE and subsequent differentiation of the PrP^{Sc} sPMCA products from those of classical BSE, which may be of value as a surveillance, diagnostic method and research tool for potentially zoonotic atypical BSE. Previously described amplification of atypical BSEs by QuIC methodology has been sensitive (between $1x10^{-6}$ and $1x10^{-7}$ for L-type and between $1x10^{-8}$ and $1x10^{-}$

^{9} for H-type) but at these dilutions discrimination of the two is not possible (Orrú et al. 2015a; Orrú et al. 2015). Discrimination of between classical, H-type and L-type BSE however can only be accomplished with much less diluted brain homogenates and subsequently poorer sensitivity (at brain dilutions between 10^{-3} and 10^{-5}) (Masujin et al., 2016).

Under sPMCA conditions found to be efficient for the amplification of H-type BSE (40 seconds of sonication time, bovine substrate and digestions of products with 10 µg/ml PK) and was detected down to 1 pg of brain material (a 10^{-10} dilution), more sensitive than the previously mentioned RT-QuIC assays. RT-QuIC measures the amyloid seeding ability of PrP^{Sc} but does not produce de novo infectious PrP^{Sc} unlike PMCA (Weber et al., 2007). This advantage of sPMCA allowed newly generated H-type PrP^{Sc} to be compared to sPMCA amplified classical BSE PrP^{Sc} produced under the same conditions. The PrP^{Sc} from sPMCA reactions seeded with a high spike (100 µg) or a low spike (1 ng of H-type brain material, 100 ng of classical BSE brain material) could be differentiated using a number of molecular characteristics which also represent the faithful propagation of the properties of the seed in sPMCA implication. The estimated molecular weight of the unlgycosylated protein of the sPMCA products was significantly larger than those seeded with classical BSE and analysed in western blotting. Western blotting also revealed that the sPMCA of H-type react with the primary antibody P4, a property not found in classical BSE PrP^{Sc} and as such has been used to strain type in the past (Biacabe et al., 2004). H-type BSE has previously been shown to be more susceptible to protease degradation than classical BSE, while the susceptibility was even more prominent in the PrP^{Sc} generated in sPMCA (Jacobs et al. 2007). While the sPMCA reactions seeded with low spikes were not on the limit of detection for this assay there is no reason to suspect successful amplification of PrP^{Sc} when spiked with just 1 ng of brain material (lowest H-type BSE amplified) would not display the previously mentioned molecular characteristics which would enable its discrimination from classical BSE.

The sPMCA method described here may not have the required sensitivity to detect to be applied to L-type BSE, certainly the sensitivity and subsequent discrimination of both H-type and L-type BSE make it suitable for further investigation as a surveillance tool. Incidence of H-type BSE has been reported due to active surveillance of primarily asymptomatic animals and has been likened to spontaneous TSE infections in humans, therefore the incidence of H-type BSE is unknown and sensitive tests able to quickly identify H-type BSE are required (Seuberlich et al., 2010). Application of sPMCA optimised for H-type amplification coupled with post amplification PrP^{Sc} discrimination would provide a surveillance method with greater sensitivity currently offered by other *in vitro* amplification techniques (Orrú et al. 2015; Masujin et al. 2016).

The identification of these atypical BSE isolates with good sensitivity and the description of their incidence in cattle herds used as food stuffs is particularly important when considering the zoonotic

threat of these isolates may be larger than that posed by classical BSE as assessed by several infection models (Béringue et al., 2008; Comoy et al., 2008; Kong et al., 2008). Furthermore the high sensitivity of the assay may enable detection of H-type BSE PrP^{Sc} in early stages of disease, while application of the technique to different bovine tissue of experimental infection may provide important insight in the tissue distribution of PrP^{Sc} during different stages of disease.

Chapter 5. Development and testing of a serial PMCA assay to specifically identify BSE in the presence of excess amounts of scrapie.

5.1. Introduction

Despite intense surveillance in the UK sheep herd no natural BSE has ever been detected in an ovine host, and only 2 natural cases of BSE in goats have been reported (Eloit et al., 2005; Jeffrey et al., 2006b). This is despite surveillance testing for BSE being carried out primarily because small ruminants were fed the same infected feed stuff that caused the spread of BSE in cattle in the epidemic of the 1980s in the UK and because sheep are susceptible to experimental BSE infections via oral challenge (Foster et al., 1993).

It is possible that this extremely low incidence of BSE in small ruminants is a result of subclinical BSE infections that current techniques do not have the required sensitivity to detect. Additionally, the lack of observed BSE could be related to the endemic scrapie infections that was also prevalent during the BSE epidemic in cattle. As a result of the prevalence of scrapie during this time, coinfections with BSE could have occurred and the presence of scrapie masked BSE from the current diagnostic techniques. Scrapie and BSE are similar prion diseases and the differentiation between the two in an ovine or caprine host can be difficult based on disease presentation and pathology in the affected animal (Gough et al., 2015). As such, definitive diagnosis depends on the analysis of the biochemical features of PrP^{Sc} and through infection studies in animal bioassays using small rodents (Bruce et al., 1994, 1991; Gough et al., 2015; Jeffrey et al., 2006a).

Animal bioassays are the current gold standard for strain typing of unknown TSE isolates, while these assays have the ability to differentiate between BSE and scrapie infections and can discriminate between different scrapie isolates, they are expensive and require years to complete. Biochemical analysis such as western blotting with various antibody combinations can differentiate between PrP^{Sc} derived from BSE and scrapie isolates (Jacobs et al., 2011; Jeffrey et al., 2006a; Langeveld et al., 2011). However the sensitivity of these assays are low and dependent on the concentration of PrP^{Sc} in the initial sample. Additionally, these assays have been developed to strain type 'pure isolates' rather than strains within mixed infections.

There is a need for a sensitive and specific diagnostic technique for detecting the presence of BSE prion in an excess scrapie prion which may be the case for coinfections of small ruminants. Previous work conducted by researchers at the University of Nottingham led to the production of an *in vitro* serial PMCA assay with the ability to detect minute seeds of BSE within excess amounts of scrapie PrP^{Sc} which would allow for the testing of subclinical infections of BSE, including in animals with dominant scrapie infections (Gough et al., 2014). Building upon this foundation, a panel of blinded samples were tested to find the sensitivity and specificity of the assay. Samples were scrapie isolates from a range of genotypes and strains, and mixtures of ovine BSE in various scrapie strains. The samples were provided by the AHPA and were tested at the APHA by strain typing techniques

recommended by the European Union reference laboratory allowing a direct comparison of the PMCA method to current methodologies to detect BSE in mixed TSE infections in sheep.

5.2. Results

5.1.1. The limit of detection of serial PMCA for ovine BSE within excess scrapie

An assay with surveillance or diagnostic purpose must have defined limits of detection. To assess sensitivity and specificity, 3 isolates of ovine BSE 10% (w/v) brain homogenates were diluted into 3 10% (w/v) scrapie positive brain homogenates (table 5.1) over dilutions of BSE from 10 to 0.001%. These dilutions were then used as spike in the serial PMCA technique to selectively amplify BSE PrP^{Sc} as described previously (Chapter 2.5). The premise of the assay relies on the fact that usually only BSE PrP^{Sc} can replicate efficiently as the substrate for conversion is switched between genotypes AHQ/AHQ and VRQ/VRQ at each round of PMCA. The PMCA products after 5 rounds amplification were analysed via SDS-PAGE electrophoresis and western blotting using SHa31 as a primary antibody to detect BSE (figure 5.1).

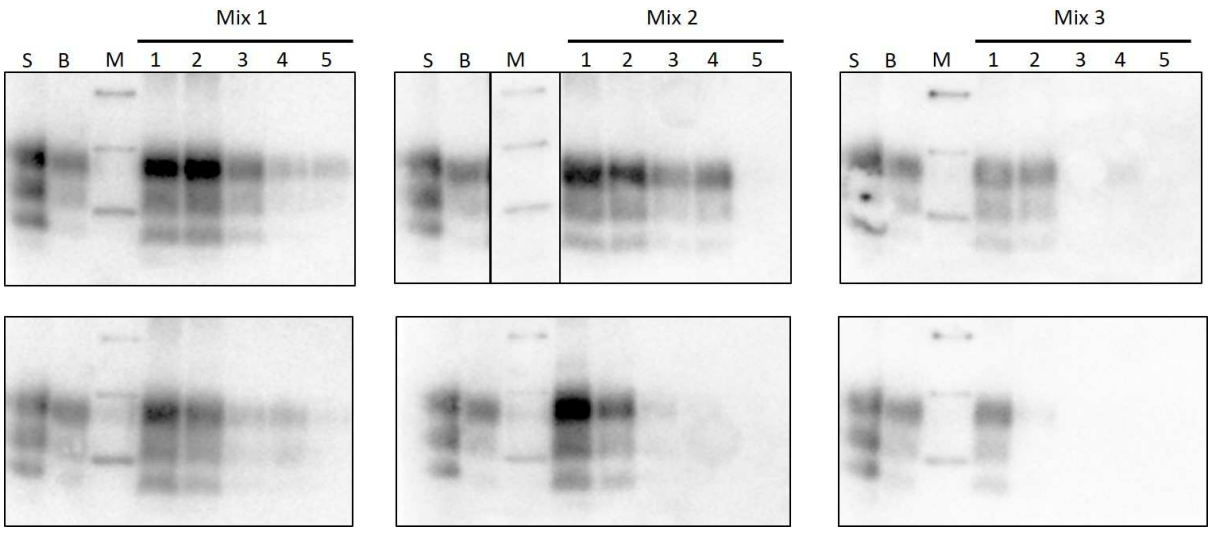


Figure 5.1. Limit of detection of ovine BSE in spikes containing excess scrapie in sPMCA assay. Three mixes of ovine BSE and scrapie brain homogenates were spiked into sPMCA reactions, for a total volume of 1 mg of brain material. Lanes consisted of scrapie (**S**) and ovine BSE (**B**) blotting controls, markers (**M**), and mixes containing 10% (**1**), 1% (**2**), 0.1% (**3**), 0.01% (**4**) and 0.001% (**5**) ovine BSE in the sPMCA spike. Ovine BSE and scrapie identities; Ovine BSE PG0137/12 and PG1500/02 (mix 1), ovine BSE PG0325/10 and scrapie PG1275/02 (mix 2), ovine BSE PG0326/12 and scrapie PG0876/04 (mix 3). Spikes were amplified for 5 rounds, alternating substrates between AHQ/AHQ (PG0210/12) and VRQ/VRQ (PG0143/12) *PRNP* genotypes at each round of amplification. Products of amplification were digested with 50 µg/ml PK, separated in SDS-Page electrophoresis, transferred to PVDF membranes and probed with primary antibody SHa31.

Table 5.1. Limit of detection of ovine BSE in BSE specific serial PMCA amplified in the presence of excess scrapie.

Mix	Isolate	Genotype	Positive replicates at BSE spike content[a]				
			10%	1.0%	0.1%	0.01%	0.001%
1	Ovine BSE - PG0137/12	AFRQ/ARQ	2/2	2/2	2/2*	2/2*	1/2*
	Scrapie - PG1500/02[b]	ARQ/ARQ					
2	Ovine BSE - PG0325/10	AHQ/AHQ	2/2	2/2	2/2*	1/2	0/2
	Scrapie - PG1275/02[b]	VRQ/VRQ					
3	Ovine BSE - PG0326/12	AHQ/AHQ	2/2	1/2	0/2	0/2	0/2
	Scrapie - PG0876/04[b]	AHQ/AHQ					

a) The number of each combination of BSE and scrapie which were positive for PrP^{Sc} following amplification. 10 µl of each mix at every dilution was spiked into 90 µl of substrate and amplified in PMCA for 5 rounds. 10% samples contained 1 µl of 10% (w/v) BSE brain homogenate (the equivalent of 100 µg of brain tissue) and 9 µl of 10% (w/v) scrapie brain homogenate (900 µg of brain tissue).

b) 10 µl of each scrapie from mix 1, 2 and 3 was assayed without BSE in triplicate to serve as a negative control. None of these controls issued detectable PrP^{Sc}.

* denotes weak positive signal.

The PMCA assay successfully detected 10 µg of ovine BSE brain material that comprised 1% of the total brain content when diluted into scrapie positive brain homogenates for all 3 isolates tested, which represented a range of host genotypes for PrP (table 5.1). The sensitivity varied with the mixtures that were used as spike with the sensitivity of the assay ranging from 1% BSE brain content for mixture 3 to 0.001% for mixture 1. The specificity of the assay was 100%, the scrapie isolates in the mixtures were amplified in triplicate in the PMCA without BSE and were negative for PrP^{Sc} after amplification. Furthermore the positive results observed produced a classical BSE profile on western blots in terms of both the molecular weight of PrP^{Sc} and dominant diglycan content (scrapie controls display more even ratios of mono-glycosylated and di-glycosylated protein) (figure 5.1).

5.1.2. Testing substrate for conversion in the PMCA assay

PMCA employs TSE free 10% (w/v) brain homogenates as a source of PrP for PMCA techniques. These brain homogenates are kept dedicated to particular users and assays, as there is subtle variation between homogenates made from different animals that affect the replication efficiency of PMCA and they are a limited resource within the laboratory. The limit of detection detailed above (table 5.1) was

performed in a substrate pair (AHQ/AHQ - PG0210/12, VRQ/VRQ - PG0143/12) not previously used for this assay (Gough et al., 2014). The sensitivity of this substrate pair was then compared to that of the pair originally used in the published assay (AHQ/AHQ- PG0209/12, VRQ/VRQ - PG1384/10) by amplifying the same spikes and visualising in western blotting after probing with SHa31 primary antibody (figure 5.2, table 5.2).

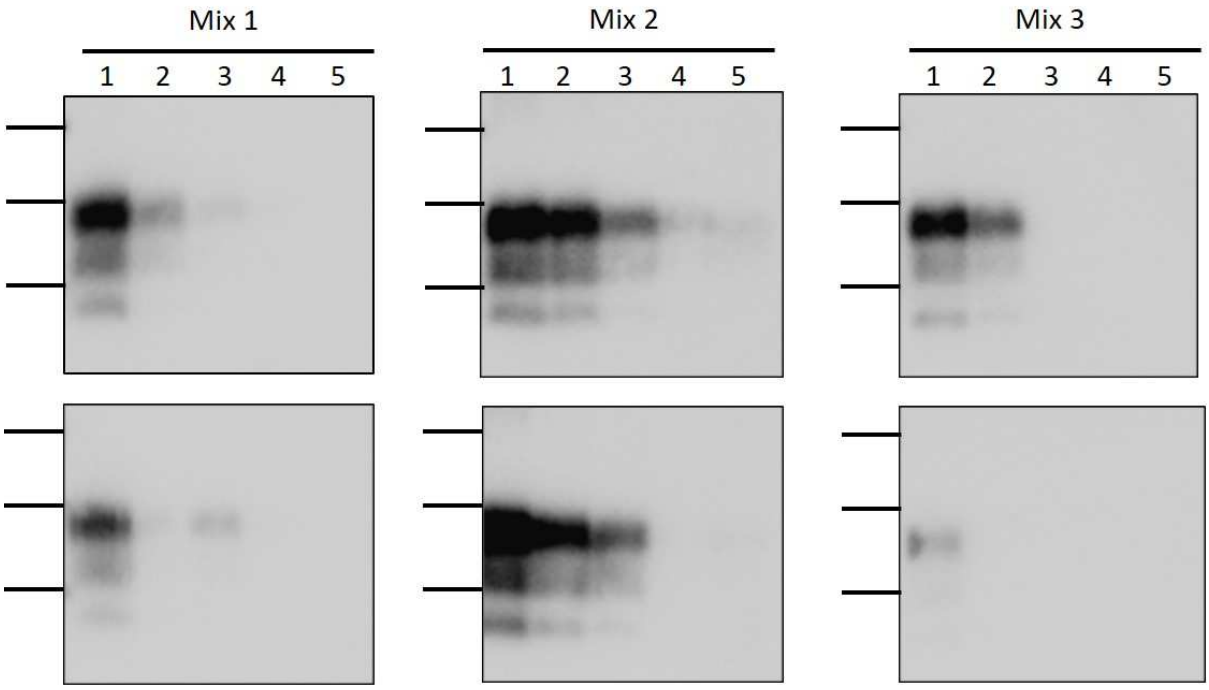


Figure 5.2. Limit of detection of ovine BSE in spikes containing excess scrapie in sPMCA assay to compare TSE free brain homogenates as substrate. Three mixes of ovine BSE and scrapie brain homogenates were spiked into sPMCA reactions, for a total volume of 1 mg of brain material. Lanes consisted of mixes containing 10% (**1**), 1% (**2**), 0.1% (**3**), 0.01% (**4**) and 0.001% (**5**) ovine BSE in the sPMCA spike. Markers of 40, 30 and 20 are indicated to the left of each blot. Ovine BSE and scrapie identities; Ovine BSE PG0137/12 and PG1500/02 (mix 1), ovine BSE PG0325/10 and scrapie PG1275/02 (mix 2), ovine BSE PG0326/12 and scrapie PG0876/04 (mix 3). Spikes were amplified for 5 rounds, alternating substrates between AHQ/AHQ (PG0209/12) and VRQ/VRQ (PG1348/10) *PRNP* genotypes at each round of amplification. Products of amplification were digested with 50 µg/ml PK, separated in SDS-Page electrophoresis, transferred to PVDF membranes and probed with primary antibody SHa31.

Table 5.2. Limit of detection of ovine BSE in BSE specific serial PMCA amplified in the presence of excess scrapie using substrates PG0209/12 and PG1384/10.

Mix	Isolate	Genotype	Positive replicates at BSE dilution into scrapie				
			10%	1.0%	0.1%	0.01%	0.001%
1	Ovine BSE - PG0137/12	AFRQ/ARQ	2/2	1/2*	2/2*	0/2	0/2
	Scrapie - PG1500/02	ARQ/ARQ					
2	Ovine BSE - PG0325/10	AHQ/AHQ	2/2	2/2	2/2	1/2*	0/2
	Scrapie - PG1275/02	VRQ/VRQ					
3	Ovine BSE - PG0326/12	AHQ/AHQ	2/2	1/2	0/2	0/2	0/2
	Scrapie - PG0876/04	AHQ/AHQ					

a) The number of each combination of BSE and scrapie which were positive for PrP^{Sc} following amplification. 10 µl of each mix at every dilution was spiked into 90 µl of substrate and amplified in PMCA for 5 rounds. 10% samples contained 1 µl of 10% (w/v) BSE brain homogenate (the equivalent of 100 µg of brain tissue) and 9 µl of 10% (w/v) scrapie brain homogenate (900 µg of brain tissue).

b) 10 µl of each scrapie from mix 1, 2 and 3 was assayed without BSE in triplicate to serve as a negative control. None of these controls issued detectable PrP^{Sc}.

* denotes weak positive signal.

The two substrate pairs had comparable sensitivity when amplifying the mixtures of ovine BSE and scrapie mixtures 2 and 3. However the substrates used to generate data in table 5.1 (PG0210/12 and PG0143/12) were 1000 times more sensitive in detecting BSE in mix one, 10 times more sensitive for mix two and comparable for mix 3, than the other pair tested (PG0209/12 and PG1348/10) (table 5.2).

5.1.3. In depth sensitivity and specificity testing in blind and double blind trial

In order to use the assay as an analytic tool and apply it to actual mixed infection samples the robustness must be verified and the sensitivity and specificity confirmed, over a wide range of isolates for ovine BSE and scrapie, including scrapie from a range of genotypes, along with samples of scrapie only.

Two cohorts of samples were included in this study and were assayed as one large experimental set. Cohorts 1 and 2 samples were single blinded. Cohort 1 consisted of a range of classical and atypical scrapie isolates from sheep with a range of genotypes for *PRNP* to further test the specificity of the assay (table 5.3). Cohort 2 was provided by the APHA, an ovine BSE brain homogenate was mixed with 3 classical scrapie isolates and an atypical scrapie isolate in various mixtures of 99%, 90%, 75%, 50%, 25%, 10%, 1%, 0.1% and 0.01% ovine BSE. Classical isolates were from animals with genotypes of VRQ/VRQ and ARQ/ARQ for *PRNP* (at amino acid positions 136, 154 and 171), while the 1-4-7 scrapie

was from an ARQ/ARQ animal and the atypical scrapie from an AHQ/ARQ animal. 1-4-7 scrapie strain in sheep produces a lesion profile with spikes in the brain regions termed 1, 4 and 7 (relating to dorsal medulla nuclei, hypothalamus, and septal nuclei of the paraterminal body) in a standard strain typing assay which is a profile consistent with BSE infections (Beck et al., 2010). The neat brain homogenates of the ovine BSE and scrapie isolates were also included in this cohort. Importantly, cohort 2 was also analysed by APHA using the EU statutory tests for detecting BSE in small ruminants thereby allowing the direct comparison of the PMCA method to existing strain typing assays.

Table 5.3. Cohort 1, classical and atypical scrapie isolates tested in the assay.

Isolate	Genotype[a]	No. of isolates[b]	No. of replicates[c]
Classical scrapie	AHQ/AHQ	1	2
	AHQ/ARQ	3	5
	AHQ/VRQ	4	5
	ARH/ARH	4	5
	ARQ/ARH	4	7
	ARQ/ARQ	2	4
	ARQ/VRQ	2	3
	VRQ/ARH	3	4
	VRQ/ARR	4	5
Atypical scrapie	ARQ/AHQ	1	2
	ARQ/ARQ	1	2
Total		29	44

a) Genotype refers to amino acids at positions 136, 154 and 171.
b) Number of individual scrapie isolates included for each genotype.
c) How many total replicates of each genotype were included in the assay.

Samples from cohorts 1 and 2 were independently blinded, 10 µl of each sample was then spiked into sPMCA substrate and amplified in duplicate for 5 rounds. After 5 rounds of amplification the sPMCA products were digested with 50 µg/ml proteinase K prior to SDS-PAGE electrophoresis. Separated proteins were transferred to PVDF blotting membrane and probed with core antibody SHa31. SHa31 recognises epitopes present in both scrapie and BSE PrP^{Sc}. sPMCA products were considered a positive reaction if the SHa31 signal exceeded a threshold densitometry value, three standard deviations from the mean of 50 replicates of scrapie amplified in sPMCA, which were western blotted and probed with SHa31.

A single positive replicate from each blinded sample was then digested and blotted to confirm the positive reaction. If the second analysis was not positive, sPMCA products were blotted a third time and the consensus of the three repeats for SHa31 reactivity taken. SHa31 positive samples were probed with P4 antibody (figure 5.3). P4 recognises an N-terminal epitope, which is present in scrapie PrP^{Sc} but is mostly lost in BSE PrP^{Sc} (Jeffrey et al., 2006b). Therefore sPMCA products were determined to have BSE PrP^{Sc} present by analysing the ratio of reactivity between these two antibodies and comparing this to previously generated ratios for control BSE and scrapie brain homogenates. After the sPMCA and all analysis were complete for all samples, the identity of the samples was revealed (tables 5.4 and 5.5).

Table 5.4. Number of BSE positive replicates found in cohort 1 for scrapie only samples.

Isolate	Genotype[a]	No. of isolates[b]	No. of replicates[c]	No. BSE positive replicates[d]
Classical scrapie	AHQ/AHQ	1	2	0
	AHQ/ARQ	3	5	0
	AHQ/VRQ	4	5	0
	ARH/ARH	4	5	0
	ARQ/ARH	4	7	0
	ARQ/ARQ	2	4	0
	ARQ/VRQ	2	3	0
	VRQ/ARH	3	4	0
	VRQ/ARR	4	5	0
Atypical scrapie	ARQ/AHQ	1	2	0
	ARQ/ARQ	1	2	0
Total		29	44	0

a) Genotype refers to amino acids at positions 136, 154 and 171.
b) Number of individual scrapie isolates included for each genotype.
c) How many total replicates of each genotype were included in the assay.
d) Number of replicates exceeding western blotting background threshold value after probing with SHa31 primary antibody.

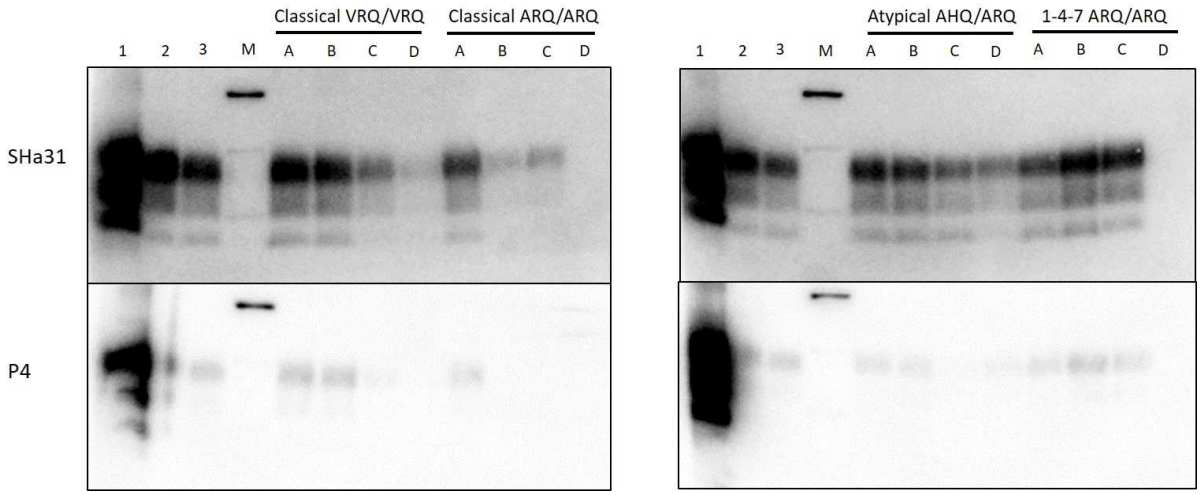


Figure 5.3. Discriminatory western blotting of sPMCA reactions spiked with ovine BSE diluted in scrapie isolates. Ovine BSE diluted in either classical VRQ/VRQ, classical ARQ/ARQ, atypical AHQ/ARQ or 1-4-7 ARQ/ARQ scrapie as indicated. Lanes consist of; scrapie (**1**) and ovine BSE (**2**) blotting controls, sPMCA amplified ovine BSE (**3**), molecular weight markers (**M**) and ovine BSE mixed with BSE at various concentrations 10% (**A**), 1.0% (**B**), 0.1% (**C**) and 0.01% (**D**). Mixes were amplified for 5 rounds of sPMCA, the substrate was alternated between AHQ/AHQ and VRQ/VRQ *PRNP* genotype at each round of amplification. sPMCA products were digested with 50 µg/ml PK before being separated in SDS-PAGE electrophoresis, transferred to PVDF membrane and probed with SHa31 or P4 primary antibodies as indicated. These blots are representative for discriminatory western blotting applied to blinded samples.

Table 5.5. Number of BSE positive replicates found in cohort 2 for BSE and scrapie mixture combined *in vitro*.

Isolate	Genotype	Ovine BSE Content (%)	No. of replicates	No. BSE positive replicates
Ovine BSE	N/A	100	1	1
Classical scrapie	ARQ/ARQ	N/A	1	0
		99	1	1
		90	1	1
		75	1	1
		50	1	1
		25	1	1
		10	2	2
		1	2	2
		0.1	1	1
		0.01	1	0
Classical scrapie	VRQ/VRQ	N/A	1	0
		99	1	1
		90	1	1
		75	1	1
		50	1	1
		25	1	1
		10	2	2
		1	2	2
		0.1	1	1
		0.01	1	0
Classical 1-4-7 scrapie	ARQ/ARQ	N/A	1	0
		99	1	1
		90	1	1
		75	1	1
		50	1	1
		25	1	1
		10	2	2
		1	2	2
		0.1	1	1
		0.01	1	0
Atypical scrapie	AHQ/ARQ	N/A	1	0
		99	1	1
		90	1	1
		75	1	1
		50	1	1
		25	1	1
		10	2	2
		1	2	2
		0.1	1	1
		0.01	1	1

In cohort 1 which contained scrapie only isolates, BSE was not detected in any of the 44 replicates amplified. In cohort 2 BSE was not present in any of the scrapie only samples but was detected in

mixtures down to 0.1% ovine BSE in all scrapie sources. The sensitivity was even greater in atypical scrapie mixtures, with ovine BSE detected down 0.01%, the lowest content tested. This gave an overall specificity of 100% and sensitivity at 0.1% ovine BSE of 100% of the assay to detect ovine BSE in mixtures of scrapie.

5.3 Discussion

The experiments detailed in this chapter were undertaken and published in collaboration with researchers at the AHPA, comparing current techniques used to strain type TSEs and their possible effectiveness at identifying BSE when present in mixtures with scrapie (Simmons et al., 2015). Samples in cohort 2 were tested by a range of current *in vitro* surveillance methods and a bioassay recommended by the European reference laboratory, a summary of the results is displayed in table 5.6 (Simmons et al., 2015). The mixtures tested by each assay represent the final content of 10% (w/v) brain homogenates and are not corrected for the relative amounts of PrP^{Sc} that were mixed together. However when the total PrP^{Sc} content was measured for each brain homogenate there was no relationship between the sensitivity of any assay and the relative concentrations of BSE PrP^{Sc} in brain homogenates (Simmons et al., 2015).

Table 5.6. The BSE detection limit of each assay used to test cohort 2.

Scrapie type	**BSE Detection limit**			
	WB	ELISA	Bioassay	PMCA
Classical ARQ/ARQ	50%	75%	1%[a]	0.10%
Classical VRQ/VRQ	25%	75%	1%[a]	0.10%
Classical 1-4-7 ARQ/ARQ	50%	90%	25%	0.10%
Atypical AHQ/ARQ	10%	99%	ND[b]	0.01%

a) indicates lowest concentration tested by bioassay
b) ND – Not detected, lowest concentration tested by bioassay was 1% in this instance

Current *in vitro* methodologies used by the European Union reference laboratory and detailed in the TSE strain characterisation in small ruminants handbook includes strain discrimination via western blotting and ELISA methodologies. Western blotting was shown to be able to correctly identify the presence of BSE in scrapie mixes between 50% and 10% (table 5.6) (Simmons et al., 2015). The assay detection sensitivity varies between different scrapie isolates, which could reflect the influence of the strain of scrapie used in the mixture and/or the genotypes of the animals for PrP. This sensitivity range is comparable to similar studies published previously within our laboratory in which western blotting

could correctly identify 60% BSE when diluted with scrapie (Gough et al., 2014). These results indicate western blotting for strain typing may only be applicable to single TSE infections, as the sensitivity is not good enough to detect ovine BSE in mixed infections (Stack et al., 2002).

ELISA was also compared as a strain typing method using scrapie and BSE mixes. The ELISA described had poor sensitivity compared to the other *in vitro* techniques (western blotting and sPMCA) being able to only identify between 99% and 75% ovine BSE for different scrapie and BSE mixes (table 5.6).

The current gold standard for strain typing TSE infections are the rodent animal bioassays, in which two transgenic mouse models are used, one which favours the propagation of BSE and expresses bovine PrP (tg110) and one which favours scrapie disease progression and expresses ovine VRQ PrP (tg338). This method, approved by the European Union reference laboratory, was able to successfully identify BSE but this was highly dependent on the strain of scrapie or genotype of the animal from which it was derived. Based upon the incubation periods of disease in tg110 mice for BSE/scrapie mixes in comparison to the 'pure' ovine BSE and scrapie source, bioassay was able to positively identify ovine BSE when mixed with classical ARQ/ARQ and VRQ/VRQ scrapie at 1%, and classical 1-4-7 ARQ/ARQ scrapie at 50% but completely failed to identify ovine BSE in a 10% mixture with atypical scrapie which was tested positive for BSE by western blotting. An advantage of bioassay over *in vitro* testing methods is the detailed immunohistochemistry profiling that can be accomplished which can provide valuable insight into disease progression and strain type. The IHC data supported the incubation period data in terms of identifying BSE in mixtures (Simmons et al., 2015).

The current study used sPMCA to detect BSE prion in brain samples even in the presence of scrapie prion. sPMCA can be highly reliant on the quality of the 10% (w/v) TSE free brain homogenates used as substrate for protein misfolding reactions. The substrates used in this study for sPMCA was compared to those previously used in published studies by this research group at the University of Nottingham, and demonstrated comparable sensitivity and even superior detection of ovine BSE for some mixtures (tables 1 and 2). These data show that multiple sources of sPMCA substrates will be appropriate for the described assay.

The serial PMCA assay described in this study was more sensitive in the detection of BSE in TSE mixes than all statutory tests recommended by the European Union reference laboratory and currently used to strain type TSE infections. The assay had 100% sensitivity and specificity when BSE was present in 0.1% when mixed with all of the scrapie strains tested, and even detected 0.01% BSE in atypical scrapie, which was the lowest dilution tested for all mixtures. The statutory test with sensitivity closest to sPMCA was transgenic mouse bioassay and this is currently held as the gold standard in strain typing, however this assay was unable to detect BSE when mixed atypical scrapie. This may be a result

of the atypical scrapie PrP^{Sc} or PrP^{C} inhibiting the replication of BSE PrP^{Sc} in the bioassay model in a similar way that human recombinant PrP^{C} can inhibit *in vitro* prion replication in PMCA or stain interference between TME strains (Yuan et al. 2013; Schutt et al. 2008). This is a significant failing of the bioassay as a discriminatory method to detect BSE in scrapie must be reliable for a range of strains of scrapie and host genotypes. In contrast to this, PMCA was able to detect 0.01% BSE in this scrapie strain. The sPMCA has further advantages over bioassay in terms of implementation as a statutory test. sPMCA is far more rapid with results obtained in a matter of days or weeks rather than years for bioassay, and is also far cheaper to implement, as animals do not need to be kept alive for long periods of time. It also uses far less animals and so improves the ethical considerations for such a screening method. Moreover immunohistochemistry is used to confirm results from incubation time data but there is an element of human interpretation with immunohistochemistry, but all results from sPMCA are processed with defined densitometry methods and there is no effect of user interpretation.

Overall, the sPMCA was the most robust assay for each TSE mixture tested had far greater sensitivity than the other assays described which are recommended by the European Union reference laboratory. The sPMCA assay was proven to be 100% specific with a sensitivity of 100% at 0.1% BSE content with other practical advantages over bioassay as a surveillance technique. Previously this assay was reported to have a sensitivity of 97% when ovine BSE was present at 1% (Gough et al., 2014). The difference in sensitivity may be due to subtle changes in sPMCA between the studies. Firstly the substrate pair for conversion in this study (PG0210/12 and PG0143/12) was found to amplify PrP^{Sc} more efficiently than those used for the publication. There is also variation in efficiency of sonication horns, the amplification in this study and in the previously published study were carried out on separate sonicators.

Detecting ovine BSE PrP^{Sc} in mixed infections using current statutory tests could be problematic if the BSE content was far lower than that of scrapie. In this scenario it is feasible that an ovine BSE signal could be masked by a larger scrapie content, but the BSE PrP^{Sc} could pose a zoonotic risk none the less. Evidence for natural scrapie and BSE coinfections is limited, though this may be due to sensitivity of current detection methods, yet studies of other TSEs offer support for coinfections. A single isolate of TME has been shown to contain two distinct strains with different phenotypic diseases and natural classical and atypical scrapie infections have been described in the same ovine host (R. A. Bessen and Marsh, 1992; Mazza et al., 2010). Experimental studies in rodents, expressing murine or ovine PrP, have demonstrated that scrapie and BSE mixed infections are theoretically possible and that scrapie could mask diagnostic BSE molecular and pathological traits (Baron and Biacabe, 2001; Corda et al., 2015).

The presented results show that in theory the sPMCA has the potential to be used to detect ovine BSE in mixed infections, however it must be stressed that artificially mixed brain homogenates containing different TSEs may not reflect the complexity of actual mixed infections *in vivo.* To fully test the sensitivity and specificity of the assay it must be used to analyse the tissues of animals from experimental coinfections.

Chapter 6. *In vitro* detection of BSE prions in scrapie and BSE co-infections.

6.1 Introduction

In Chapter 5 a sPMCA based assay was shown to be more successful at detecting the presence of ovine BSE in the presence of excess scrapie compared to assays currently endorsed by the European Union reference laboratory. Not only was sPMCA more sensitive than other *in vitro* assays it also outperformed the current gold standard bioassay. The sPMCA assay was a robust method for the detection of BSE in the presence of excess scrapie, with 100% specificity of 100% sensitivity at 0.1% BSE (Simmons et al., 2015).

While these results undoubtedly represent a step forward in detection capabilities for BSE in possible co-infections, the samples tested were not genuine tissues from co-infected animals but *in vitro* mixes of 10% (w/v) brain homogenates. TSE disease progression *in vivo* is much more complicated than this simple model used to define the limits of the assay. Therefore the sPMCA assay was applied to a range of different tissue samples from sheep that were experimentally infected with both BSE and scrapie prions.

There are a number of fundamental questions this research sets out to answer. Firstly is it possible for both scrapie and BSE PrP^{Sc} to replicate independently in the same small ruminant host and is the sPMCA assay robust enough to detect them? A number of studies have documented different TSE strains replicating in the same host, for example two distinct strains of TME in hamsters and classical and atypical scrapie in sheep (R. A. Bessen and Marsh, 1992; Mazza et al., 2010). Evidence for the possibility of scrapie and BSE co-infections come from studies using two lines of mice, one inbred line and one transgenic line expressing ovine PrP, both of which demonstrated parallel scrapie and BSE disease progression and PrP^{Sc} replication (Baron and Biacabe, 2001; Corda et al., 2015).

In such mixed infections it is in the interest of human health to know in which tissues PrP^{Sc} is deposited and if the localisation differs for scrapie and BSE. It is also important to know if in co-infection a dominant strain can mask the presence of others. This information can be used to inform health and safety and best practices within regard to which tissues from small ruminants should enter the human food chain.

This study applied the sPMCA assay (detailed in chapter 5) to sheep with genotypes of ARQ/ARQ and VRQ/ARQ, at amino acid positions 136, 154 and 171 in the *PRNP* gene, which had been experimentally infected with either scrapie sheep brain pool number 1 (SSBP/1) scrapie, BSE or both. The animals that were inoculated with SSBP/1 and BSE were done so at the same time or with the BSE administered 3 months later. In addition to these experimental studies, a flock of sheep with endemic scrapie infection in which scrapie is believed to be contracted naturally early in life and develops into clinical disease at 2 years of age where given a 'secondary' BSE infection at either 8 or 21 months of age.

These sheep exposed to 'environmental scrapie' were all VRQ/VRQ genotype for *PRNP*. Samples of brain, spleen and prescapular lymph node were taken from animals in all test groups. These tissues were processed into 10% (w/v) homogenates (chapter 2.3.3) before testing in the sPMCA assay for the presence of BSE PrP^{Sc}.

6.2 Results

6.2.1. Application of sPMCA to different tissue types.

Previously sPMCA has been used to detect BSE prions in mixtures of BSE and scrapie brain homogenates, however the technique has not been used on other tissue types. PMCA may be inhibited by the sample matrix of different tissue types, for example blood vessels and the dura mater (or meninges) (membranous sack enveloping the brain) must be removed before a brain can be processed as a 10% (w/v) brain homogenate to provide an effective substrate for PMCA. It was therefore necessary to test whether spleen or prescapular lymph nodes were suitable as spikes and did not produce a detrimental matrix effect within PMCA reactions.

To determine if the sensitivity of the sPMCA to spleen and prescapular lymph nodes samples was comparable to that of brain tissues, a dilution series of BSE was spiked into sPMCA reactions which contained only brain tissue, or 10 µl of 10% (w/v) spleen or lymph node homogenate (figure 6.1), tissues were processed using a bead beater (chapter 2.3.3). No inhibitory effects due to the presence of lymph node homogenate on PMCA were seen even when the ovine BSE spike was only 0.1 µg (figure 5.1C). At the lowest levels of BSE spike (0.1 µg) the reactions containing spleen only amplified 1 of 2 samples (figure 5.1B) which was less efficient than those containing brain or lymph nodes, however this effect was not noticed when the spleen had been processed by hand using syringe and blunt needles (figure 5.1D).

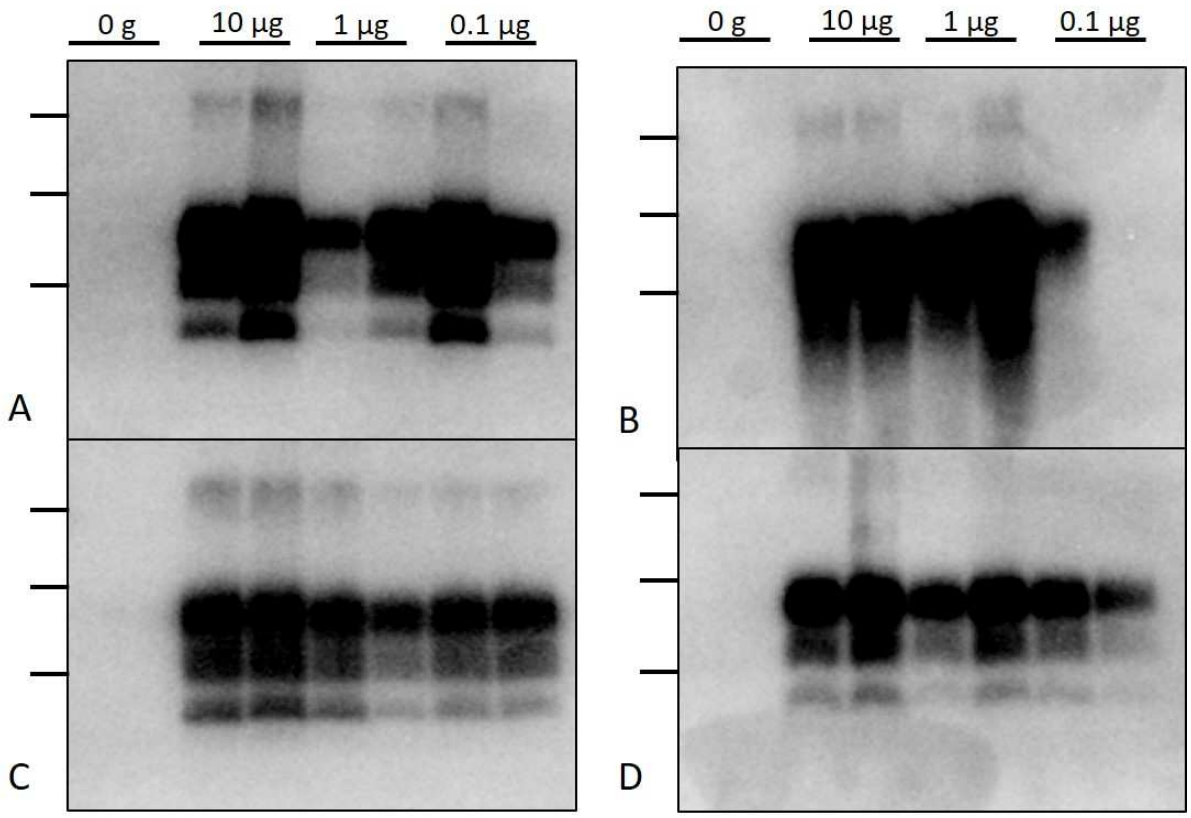


Figure 6.1 Sensitivity of sPMCA in the presence of brain, spleen and prescapular lymph node tissue homogenate. Tissue samples were processed in a bead beater (A-C) or were syringe processed (D). An isolate of ovine BSE (PG0325/10) was spiked at 10 , 1 and 0.1 µg into a PMCA reaction containing 10 µl of 10% (w/v) ovine homogenate of either brain (A), spleen (B, D) or lymph node (C). Markers indicated at 40, 30 and 20 kDa. Spikes were amplified in alternating substrates for a total of 5 rounds. PMCA products were then digested with proteinase K, separated in SDS-PAGE, western blotted and probed with SHa31 primary antibody.

6.2.2. Application of sPMCA to ovine tissue samples from co-infected animals

The sensitivity and specificity of the sPMCA assay based on *in vitro* mixes of BSE and scrapie brain homogenates, in addition to the testing of the assay with different tissue types, proved the assay was robust and suitable for testing genuine co-infection samples. All co-infection animal experiments were carried out at the Roslin Insitute by Professor Nora Hunter. The resulting samples were from three genotypes of sheep ARQ/ARQ, VRQ/ARQ and VRQ/VRQ (table 6.1). The ARQ/ARQ and VRQ/ARQ Cheviot sheep were from a scrapie free flock (ARSU flock, Defra project SE1436) and received 2 ml subcutaneous injections of TSE infected 10% (w/v) brain homogenates in the hind limbs, this was either SSBP/1 scrapie, BSE or both SSBP/1 and BSE on the same day, or SSBP/1 and then BSE dosing 84 days later. Scrapie and BSE were dosed in separate injections into different hind limbs, including when both were administered on the same day. Initial dosing took place at 6 months of age, delayed

BSE dosing took place at 9 months. VRQ/VRQ genotype animals were from the Neuropathogenesis Unit (NPU) Cheviot flock which has an endemic scrapie infection, 100% of VRQ/VRQ animals in this flock develop scrapie, at around 2 years of age. BSE was administered to these animals as 2ml of infected 10% (w/v) brain homogenate in a subcutaneous injection at either 8 months or 21 months of age. Animals were culled upon observation of the first signs of clinical disease. Several methodologies were used to strain type the TSE in these animals by different researchers, as well as the sPMCA applied to brain, spleen and prescapular lymph nodes in this thesis. Brain samples were analysed in rodent bioassays at the Roslin Institute and brain and immunohistochemistry (IHC) at the AHPA.

Table 6.1 Samples from the Roslin institute assayed by sPMCA

Genotype[a]	**Inoculum**[b]	**Replicates**[c]
ARQ/ARQ	SSBP/1	2
	BSE	6
	SSBP/1 and BSE	5
	SSBP/1 then BSE	5
VRQ/ARQ	SSBP/1	2
	BSE	2
	SSBP/1 and BSE	5
	SSBP/1 then BSE	5
VRQ/VRQ	Natural Scrapie	2
	Natural scrapie then BSE	5

a- Genotype of animals at amino acid positions 136, 154 and 171.
b- Different TSEs used to challenge animals. ARQ/ARQ and VRQ/VRQ animals received initial doses at 6 months of age, secondary infections were administered 3 months later. For VRQ/VRQ, natural scrapie was an environmental infection, if inoculated with BSE this took place at 8 or 21 months of age
c- Number of animals from in each treatment group tested by sPMCA.

Brain, spleen and lymph nodes provided by the Roslin Institute were processed into 10% (w/v) brain homogenates using a bead beater as detailed in chapter 2.3.3. 10 µl of processed homogenates were amplified in PMCA for 5 rounds, the first round of which took place in AHQ/AHQ substrate and then subsequent rounds alternating between VRQ/VRQ and AHQ/AHQ substrate. The products after round 5 were digested with 50 µg/ml PK before being analysed in SDS-PAGE and western blotting. Initially blots were probed with SHa31 to find positive amplifications (figures 6.2, 6.3 and 6.4), this was carried out in duplicate (data not shown). Those ovine tissues which produced a SHa31 signal which exceeded the background signal threshold value (chapter 5) were further profiled by reactivity with SHa31 and

P4 primary antibodies (figures 6.5 and 6.6). To differentiate between BSE and scrapie PrP^{Sc} first each tissue sample had to again exceed the SHa31 background blotting value, if this was the case the ratio of signal between each antibody was calculated, which is characteristic of either scrapie or BSE and is described in chapter 5, results summarised in table 2. Any samples which were SHa31 positive on initial screening (figures 6.2, 6.3, and 6.4), but failed to exceed the blotting background threshold reactivity during characterisation western blots (figures 6.5 and 6.6) were blotted a third time to confirm if the PMCA product was positive or negative. All sample which required testing a third time failed to exceed background blotting value (figure 6.7).

The results of the assay are summarised in table 6.3. BSE was not found to be present in any of the control group that were inoculated with scrapie only (0/2, 0/2, 0/2).

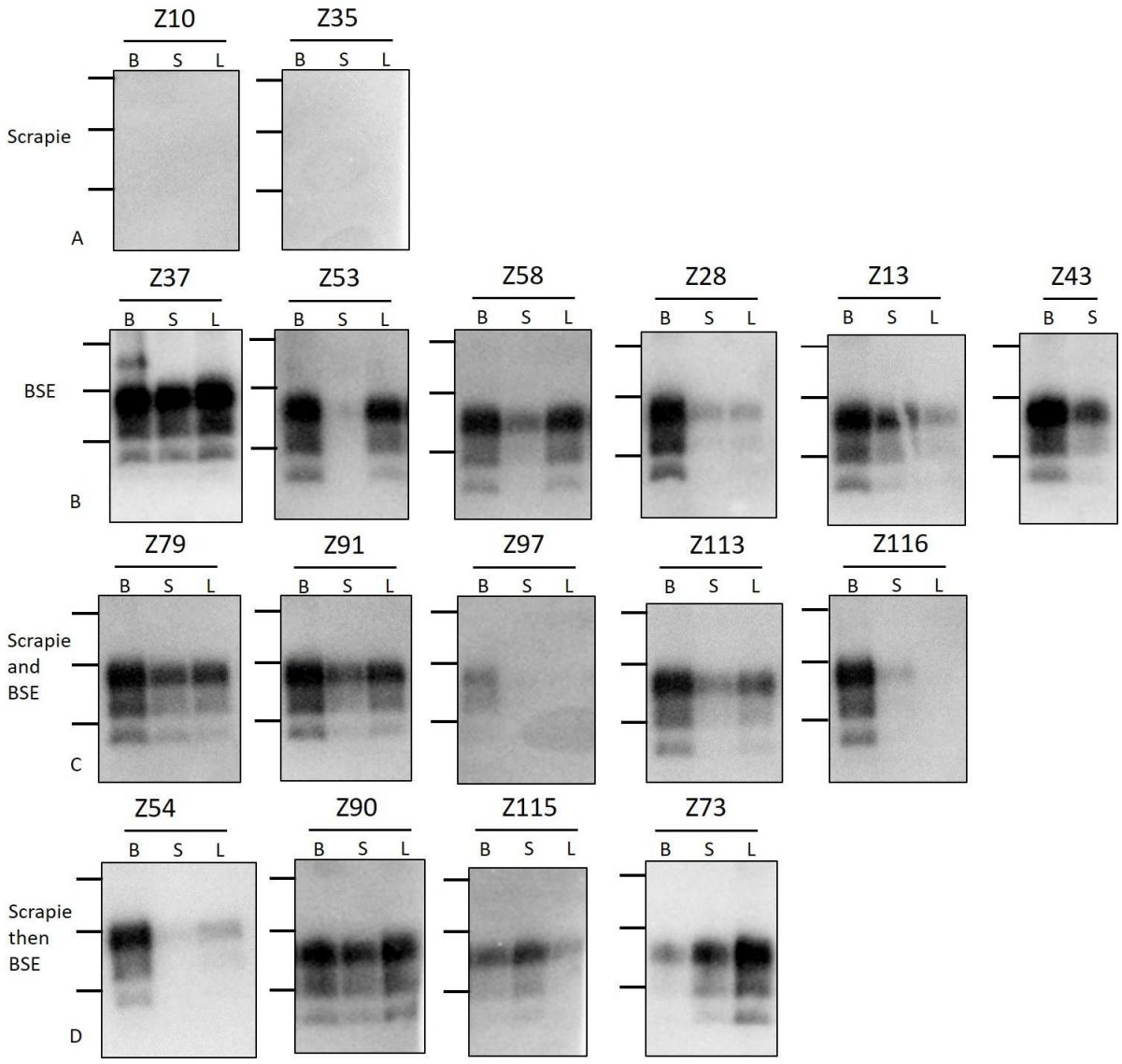


Figure 6.2 Screening of ARQ/ARQ sheep tissues for BSE PrP^{Sc}. Western blots were developed with SHa31 to test for positive sPMCA reactions that were spiked with brain (B) spleen (S) or prescapular lymph node (L) ovine tissues. Ovine tissues were derived from ARQ/ARQ animals dosed with scrapie (row A), BSE (row B), scrapie and BSE on the same day (row C) or scrapie followed by BSE 3 months later (row D). The blots are representative of the analysis carried out in duplicate for each sample. Molecular mass markers are indicated to the left of each western blot displaying 40, 30 and 20 kDa. Signals that exceeded a background threshold were subjected to further SHa31 and P4 blotting (figure 6.5)

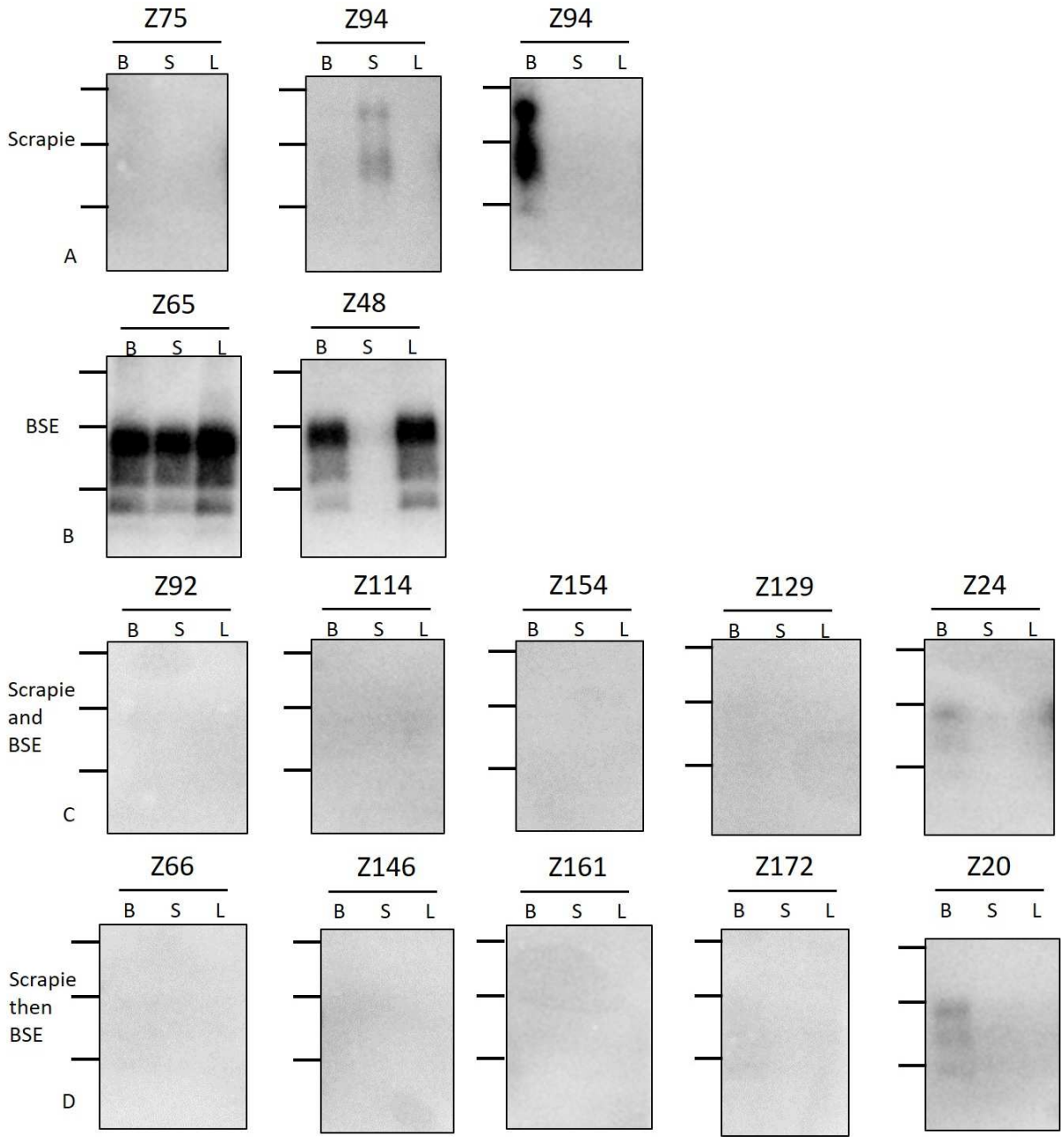


Figure 6.3 Screening of VRQ/ARQ sheep tissues for BSE PrP^{Sc}. Western blots were developed with SHa31 to test for positive sPMCA reactions that were spiked with brain (B) spleen (S) or prescapular lymph node (L) ovine tissues. Ovine tissues were derived from VRQ/ARQ animals dosed with scrapie (row A), BSE (row B), scrapie and BSE on the same day (row C) or scrapie followed by BSE 3 months later (row D). The blots are representative of the analysis carried out in duplicate for each sample. Molecular mass markers are indicated to the left of each western blot displaying 40, 30 and 20 kDa. Two blots of animal Z94 are displayed which received only scrapie inoculation, positive signal was detected in the brain and spleen of each blot. Signals that exceeded a background threshold were subjected to further SHa31 and P4 blotting (figure 6.5).

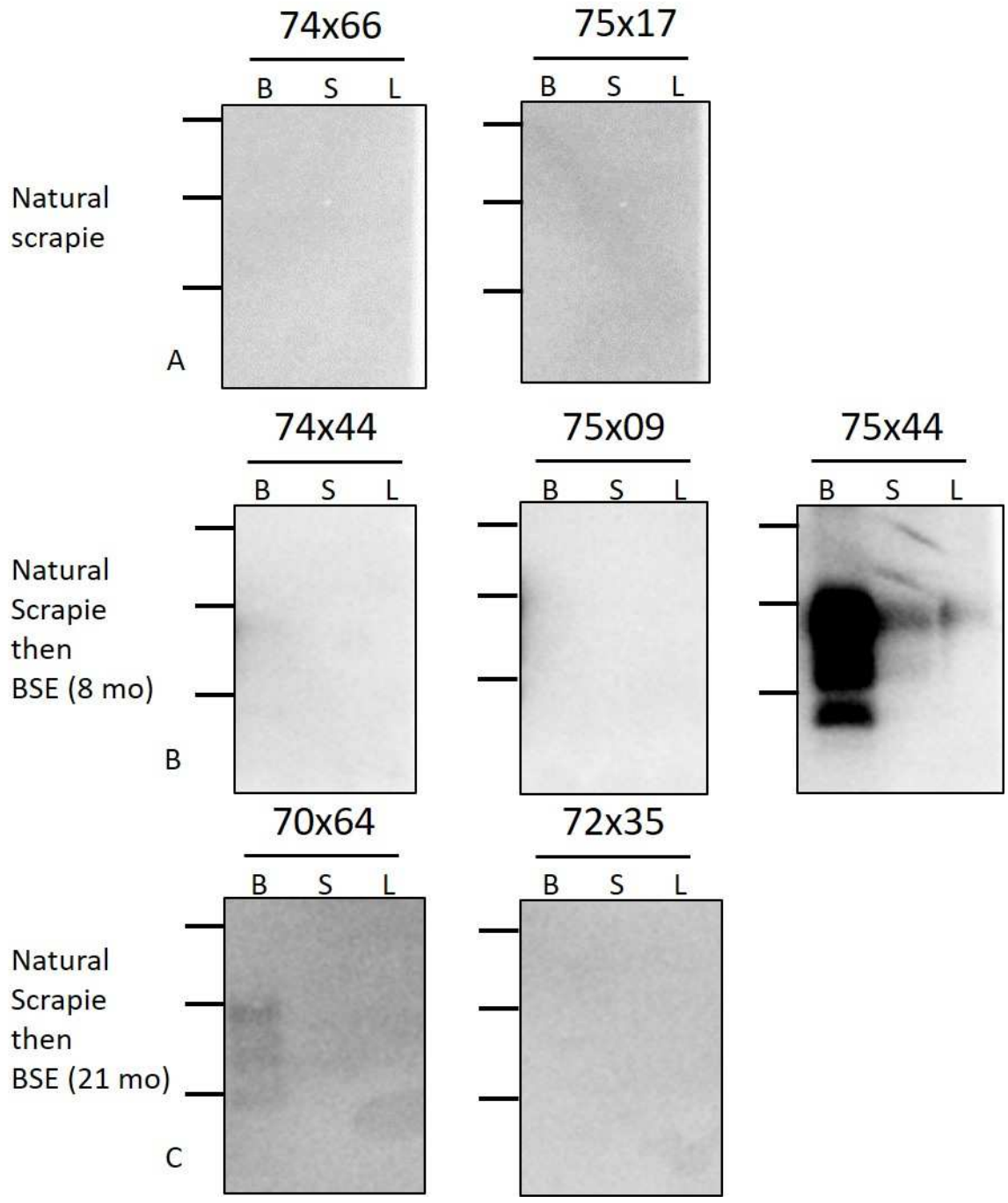


Figure 6.4 Screening of sheep from the Neuropathogenesis Unit cheviot flock for BSE PrP^{Sc}. Western blots were developed with SHa31 to test for positive sPMCA reactions that were spiked with brain (B) spleen (S) or prescapular lymph node (L) ovine tissues. Ovine tissues were derived from VRQ/VRQ (except ARQ/ARQ 75x15) animals which develop scrapie naturally due to the endemic scrapie infection in the flock (row A), also displayed are animals which were injected with BSE at 8 months (row B) and at 21 months (row C) of age. The blots are representative of the analysis carried out in duplicate for each sample. Molecular mass markers are indicated to the left of each western blot displaying 40, 30 and 20 kDa. Signals that exceeded a background threshold were subjected to further SHa31 and P4 blotting (figure 6.5).

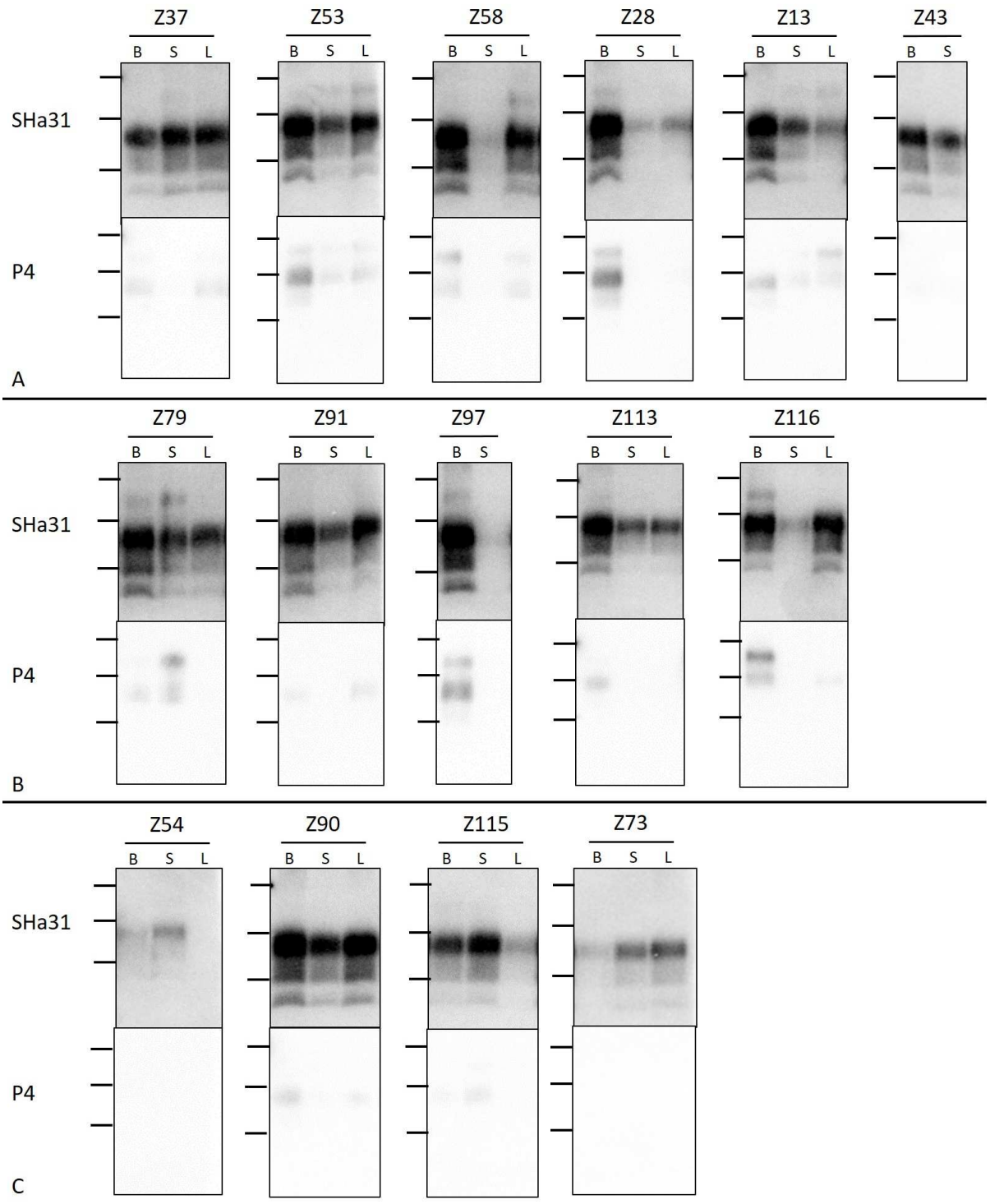


Figure 6.5 Western blots to confirm identity of sPMCA products from ARQ/ARQ animals. sPMCA Products were probed with SHa31 and P4 (as indicated) from reactions seeded with brain (B) spleen (S) or prescapular lymph nodes (L) after identification with of positive signals on initial screening (figure 3). Tissues were from animals that inoculated with BSE (row A), scrapie and BSE on the same day (row B) or scrapie followed BSE 3 months later (row C). Markers indicated at 40, 30 and 20 kDa to the left of each western blot.

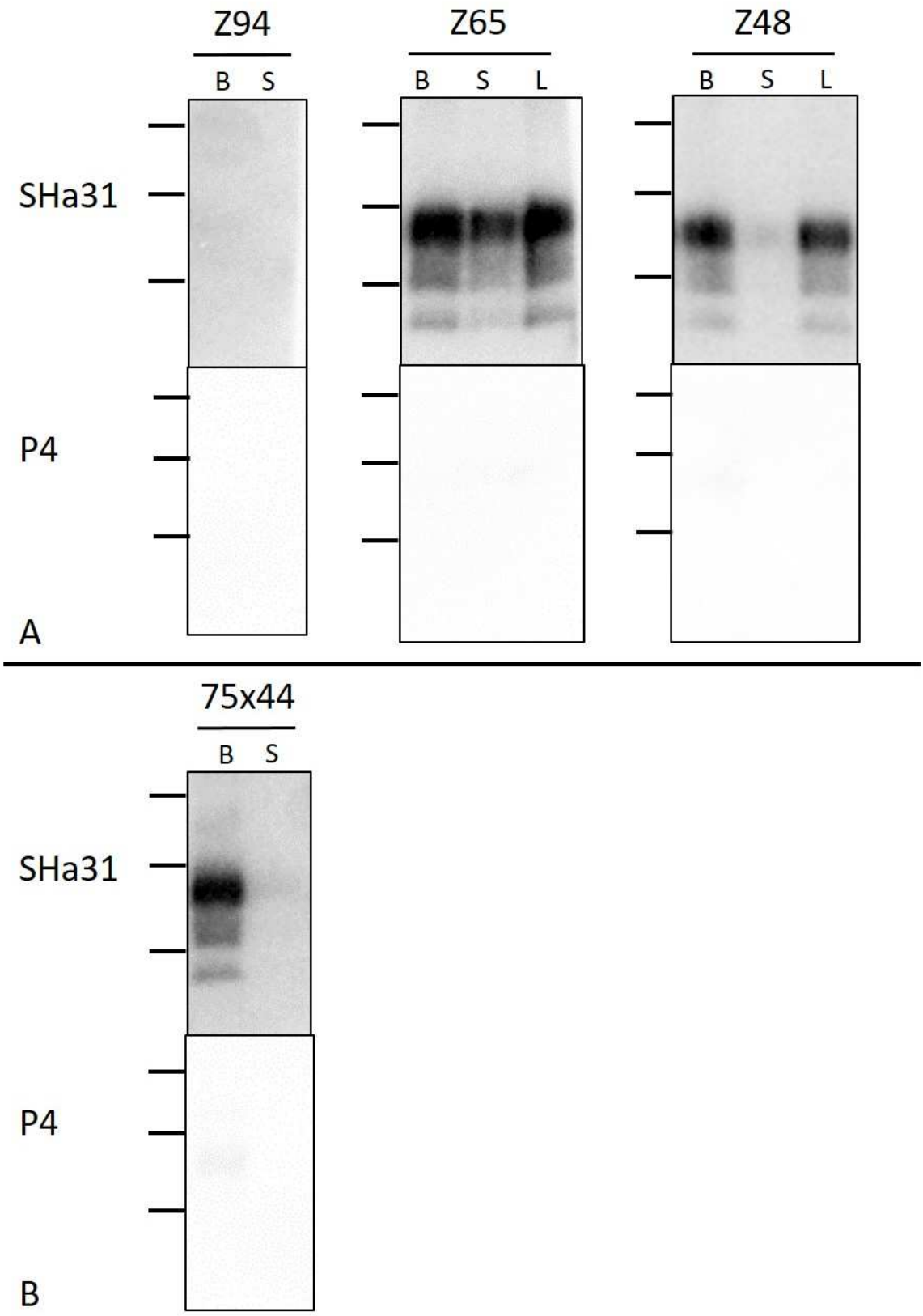


Figure 6.6 Western blots to confirm identity of sPMCA products from VRQ/ARQ and NPU cheviot flock animals. sPMCA Products were probed with SHa31 and P4 (as indicated) from reactions seeded with brain (B) spleen (S) or prescapular lymph nodes (L) after identification of

positive signals on initial screening (figure 3). VRQ/ARQ animals (row A) were inoculated with either scrapie (Z94) or BSE (Z65 and Z48). NPU cheviot sheep 75x44 (row B) was administered BSE at 8 months of age, at this age sheep have contracted endemic scrapie. Tissues were from animals that inoculated with BSE (row A), scrapie and BSE on the same day (row B) or scrapie followed BSE 3 months later (row C). Markers indicated at 40, 30 and 20 kDa to the left of each western blot.

Table 6.2 Summary of densitometry applied to ovine tissues probed with SHa31 and P4

Animal[a]	Genotype[b]	Tissue[c]	Exceeded background[d]	Ratio[e]
Z37	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z53	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z58	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z28	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	
		Lymph	Yes	BSE
Z13	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z43	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
Z79	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z91	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z97	ARQ/ARQ	Brain	Yes	BSE
		Spleen	No	
Z113	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z116	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
Z54	ARQ/ARQ	Brain	Yes	BSE
		Spleen	No	
		Lymph	No	
Z90	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z115	ARQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z73	ARQ/ARQ	Brain	No	
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z94	VRQ/ARQ	Brain	No	
		Spleen	No	
Z65	VRQ/ARQ	Brain	Yes	BSE
		Spleen	Yes	BSE
		Lymph	Yes	BSE
Z48	VRQ/ARQ	Brain	Yes	BSE
		Spleen	No	
		Lymph	Yes	BSE
75x44	VRQ/VRQ	Brain	Yes	BSE
		Spleen	No	

a) Animal identification number
b) Genotype at positions 136, 154 and 171
c) Ovine tissue tested, either brain, spleen or prescapular lymph node.
d) SHa31 signal of tissue exceed the western blotting background cut off threshold
e) Ratio of SHa31 to P4 signal identity BSE present or absent. Ratio not applied to any samples that did not exceed SHa31 background threshold

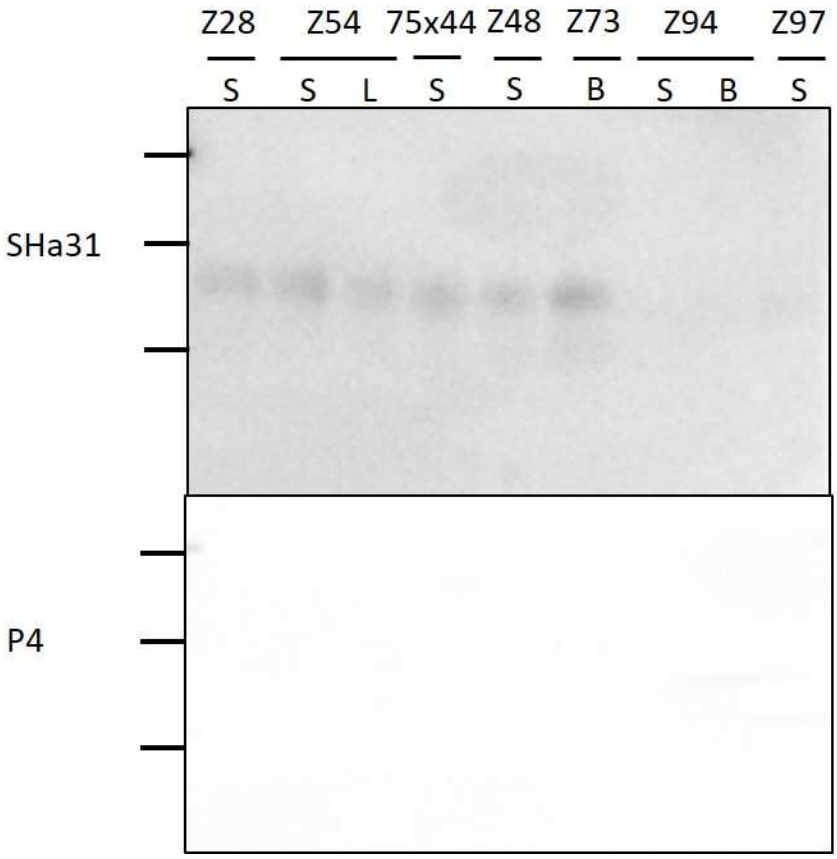


Figure 6.7 Confirmatory western blots of sPMCA products with conflicting SHa31 blots. Reactions seeded with either brain (B) spleen (S) or prescapular lymph nodes (L) which produced positive signals in initial screens (figures 2 and 3) but failed to produce positive signals on identity blotting (figures 5 and 6) were probed again with Sha31 and P4 antibodies. All SHa31 signals displayed are below western blotting background threshold. Markers indicated at 40, 30 and 20 kDa to the left of each western blot.

Table 6.3 Summary of sPMCA assay applied to experimentally infected animals

Genotype[a]	Inoculum[b]	Animal ID[c]	BSE detected[d]		
			Brain	Spleen	Lymph node
ARQ/ARQ	SSBP/1	Z10	No	No	No
		Z35	No	No	No
	BSE	Z37	Yes	Yes	Yes
		Z53	Yes	Yes	Yes
		Z58	Yes	Yes	Yes
		Z28	Yes	No	Yes
		Z13	Yes	Yes	Yes
		Z43	Yes	Yes	NT
	SSBP/1 and BSE	Z79	Yes	Yes	Yes
		Z91	Yes	Yes	Yes
		Z97	Yes	No	No
		Z113	Yes	Yes	Yes
		Z116	Yes	Yes	No
	SSBP/1 then BSE	Z54	Yes	No	No
		Z90	Yes	Yes	Yes
		Z115	Yes	Yes	Yes
		Z73	No	Yes	Yes
VRQ/ARQ	SSBP/1	Z75	No	No	No
		Z94	No	No	No
	BSE	Z65	Yes	Yes	Yes
		Z48	Yes	No	Yes
	SSBP/1 and BSE	Z92	No	No	No
		Z114	No	No	No
		Z154	No	No	No
		Z129	No	No	No
		Z24	No	No	No
	SSBP/1 then BSE	Z66	No	No	No
		Z146	No	No	No
		Z161	No	No	No
		Z172	No	No	No
		Z20	No	No	No
VRQ/VRQ	Natural scrapie	74x66	No	No	No
ARQ/ARQ	Natural scrapie	75x17	No	No	No
VRQ/VRQ	Natural scrapie then BSE[e] (8)	74x44	No	No	No
		75x09	No	No	No
		75x44	Yes	No	No
	Natural scrapie then BSE[e] (21)	70x64	No	No	No
		72x35	No	No	No

a) Genotype of animal at position 136, 154 and 171
b) TSEs that animals were inoculated with
c) Animal identification numbers
d) sPMCA detection of BSE in either brain, spleen or prescapular lymph node from infected animals. NT – not tested.
e) Animals with natural scrapie that were injected with BSE either at 8 month or 21 months age as indicated.

BSE was not detected in any of the animals that received only SSBP/1 scrapie inoculation for either ARQ/ARQ or VRQ/ARQ and animals inoculated with natural scrapie infections. When animals received BSE only it was detected in both brain and lymph node samples in all animals tested but was not detected in the spleen of 1 individual for both the ARQ/ARQ (17% of the animals) and VRQ/ARQ (50% of the animals) groups. The animals that received BSE and scrapie were then considered based on genotypes:

For the ARQ/ARQ group, of the six ARQ/ARQ animals that received only BSE all brain and lymph nodes tested positive for BSE (except spleen for Z43 which was not tested as it was not provided). In this group all spleens tested positive for BSE except in animal Z28. All 5 animals which were injected with BSE and SSBP/1 on the same day had BSE present in the brain tissue. However in 2 of 5 animals (Z97 and Z116) BSE was not present in the spleen and 1 of those (Z97) neither was it present in the prescapular lymph node. These two tissue types were positive for BSE in the remaining animals. When BSE was injected into animals 3 months after SSBP/1 and the scrapie infection was presumably already established, the transmission of BSE was lower. Animal Z54 BSE was present in the brain but absent in spleen and lymph, conversely BSE was not present in the brain of Z73 but was found in the spleen and prescapular lymph node.

BSE transmission to VRQ/ARQ animals was much less efficient, whilst animals inoculated with BSE only consistently had BSE prion detectable in brain and lymph tissue, BSE was not found to be present in any tissues when SSBP/1 was administered either on the same day (0/5) or 3 months before BSE (0/5). Sheep from the NPU flock with endemic natural scrapie infections followed by challenge with BSE at 8 or 21 months of age did not develop BSE as judged by the detection of BSE prions by the sPMCA assay in all but one animal. Sheep 75x44 (challenged with BSE at 8 months of age) had detectable BSE prions in the brain but no BSE was found in the spleen or lymph nodes, indicating mixed infection in this genotype is possible and may be more permissible than in the heterozygotes.

6.3 Discussion

The sPMCA assay's output is the detection, or absence, of BSE and does not confirm the presence of other TSEs in a mixed infection, and therefore cannot qualify a mixed infection alone. To fully interpret the results of applying this sPMCA assay to the *in vivo* mixed infections summarised in tables 6.4 and 6.5 it is necessary to first acknowledge and discuss the conventional strain typing methods that were also applied to the same samples. These assays were western blotting of brain tissues, antibody epitope mapping of PrP^{Sc}, PrP^{Sc} distribution profiling of brain regions, passage of prion in mouse

bioassays, as well as observations of attack rate and incubation times in the TSE infected sheep. These conventional assays were all conducted by external collaborators (APHA and the Roslin Institute).

A summary of the conventional strain typing techniques are displayed in tables 6.4 and 6.5. Animals that received both BSE and scrapie were analysed in terms of incubation period of TSEs and this was compared to scrapie and BSE only control groups. Incubation periods and lesion profiling produce reliable data to identity the infectious TSE agent strain but the effects on incubation times of mixed infections are as yet unknown (Houston et al., 2015). The Western blots were carried out on homogenised brain tissues from test animals, using two antibodies that react differently with BSE and scrapie, to characterise whether the PrP^{Sc} was typical of BSE or scrapie. Primary antibody 6H4 is a core antibody which has an epitopes present in both scrapie and BSE PrP^{Sc}, however the N-terminal P4 antibody epitope is present in scrapie and not BSE (Jeffrey et al., 2006a). The pattern of PrP^{Sc} dissemination throughout regions of the brain are specific for different strains of scrapie and for BSE, as such, neuroanatomical deposition profiles of PrP^{Sc} were generated to discriminate between scrapie and BSE infections (González et al., 2003). Complex immunohistochemistry was carried out on fixed brain tissues, mapping epitopes of different primary antibodies. This immunohistochemistry involved probing PrP^{Sc} with up to 6 antibodies, 3 that bound to the PrP core and 3 to the N-terminal region, scrapie PrP^{Sc} reacts with both groups whereas BSE is detected strongly with core antibodies and very weakly with N-terminal antibodies (Jeffrey and González, 2007). Brain tissue from individuals from VRQ/ARQ and ARQ/ARQ animals inoculated with both scrapie and BSE (together or with delayed BSE) was also used to intracerebrally inoculate three inbred mice strains RIII, C57BL and VM. BSE and scrapie inocula have distinct incubation times in these mice lines and the regions of the brain in which vacuolation occurs is specific for each TSE (Bruce et al., 1997).

Table 6.4 Summary of sPMCA and conventional tests applied to ovine tissues from co-infection

Genotype[a]	Inoculum[b]	Animal ID[c]	sPMCA BSE detection[d]			Western Blot[e]	PrP^{Sc} epitope mapping[f]	Neuroanatomical profile[g]
			Brain	Spleen	Lymph node	Brain	Brain	Brain
ARQ/ARQ	SSBP/1	Z10	No	No	No	Scrapie	Scrapie	Scrapie
		Z35	No	No	No	Scrapie	Scrapie	Scrapie
	BSE	Z37	Yes	Yes	Yes	BSE	BSE	BSE
		Z53	Yes	Yes	Yes	BSE	BSE	BSE
		Z58	Yes	Yes	Yes	BSE	BSE	BSE
		Z28	Yes	No	Yes	BSE	BSE	BSE
		Z13	Yes	Yes	Yes	BSE	BSE	BSE
		Z43	Yes	Yes	NT	BSE	BSE	BSE
	SSBP/1 and BSE	Z79	Yes	Yes	Yes	BSE	BSE	BSE
		Z91	Yes	Yes	Yes	BSE	BSE	BSE
		Z97	Yes	No	No	BSE	BSE	BSE
		Z113	Yes	Yes	Yes	BSE	BSE	BSE
		Z116	Yes	Yes	No	BSE	BSE	BSE
	SSBP/1 then BSE	Z54	Yes	No	No	BSE	BSE	BSE
		Z90	Yes	Yes	Yes	BSE	BSE	BSE
		Z115	Yes	Yes	Yes	Scrapie	Scrapie	Scrapie
		Z73	No	Yes	Yes	NT	NT	NT
VRQ/ARQ	SSBP/1	Z75	No	No	No	Scrapie	Scrapie	Scrapie
		Z94	No	No	No	Scrapie	Scrapie	Scrapie
	BSE	Z65	Yes	Yes	Yes	BSE	BSE	BSE
		Z48	Yes	No	Yes	BSE	BSE	BSE
	SSBP/1 and BSE	Z92	No	No	No	Scrapie	Scrapie	Scrapie
		Z114	No	No	No	Scrapie	Scrapie	Scrapie
		Z154	No	No	No	NT	NT	Scrapie
		Z129	No	No	No	NT	NT	NT
		Z24	No	No	No	NT	NT	NT
	SSBP/1 then BSE	Z66	No	No	No	Scrapie	Scrapie	Scrapie
		Z146	No	No	No	Scrapie	Scrapie	Scrapie
		Z161	No	No	No	Scrapie	Scrapie	Scrapie
		Z172	No	No	No	Scrapie	Scrapie	Scrapie
		Z20	No	No	No	NT	NT	NT
VRQ/VRQ	Natural scrapie	74x66	No	No	No	Scrapie	NT	NT
ARQ/ARQ	Natural scrapie	75x17	No	No	No	Scrapie	NT	NT
VRQ/VRQ	Natural scrapie then BSE[h] (8)	74x44	No	No	No	Scrapie	NT	NT
		75x09	No	No	No	Scrapie	NT	NT
		75x44	Yes	No	No	Scrapie	NT	NT
	Natural scrapie then BSE[h] (21)	70x64	No	No	No	Scrapie	NT	NT
		72x35	No	No	No	Scrapie	NT	NT

a) Genotype of animal at position 136, 154 and 171

b) TSEs that animals were inoculated with

c) Animal identification numbers
d) sPMCA detection of BSE in either brain, spleen or prescapular lymph node from infected animals.
e) Identity of PrP^{Sc} as tested by core (6H4) and N-terminal (P4) primary antibody probing of western blots – carried out at Roslin Institute
f) TSE strain was described by PrP^{Sc} reactivity with up to six antibodies in tissues embedded in wax – carried out by Martin Jeffrey at AHPA
g) Neuroanatomical distribution of PrP^{Sc} deposition – carried out by Martin Jeffrey at AHPA
h) Animals with natural scrapie that were injected with BSE either at 8 month or 21 months age as indicated.

NT – Not tested.

When SSBP/1 and BSE were administered to ARQ/ARQ sheep on the same day all conventional assays applied to the sheep as well as mice bioassays determined that the animals had BSE infections, and no scrapie was found to be present in these animals (tables 6.4 and 6.5). In agreement, BSE was also found to be present in the brain of all animals tested using sPMCA. Testing by sPMCA found that BSE was not present in the spleen of one animal (Z97) and lymph node of two (Z97 and Z116) but was present in all of the other LRS samples analysed from the 5 sheep.

Table 6.5 Strain typing of ovine brain tissue inoculum used to infect mouse bioassay

Genotype[a]	Inoculum[b]	Animal ID[c]	Incubation period[d]	Lesion profiles[e]	Western blot[f]
ARQ/ARQ	SSBP/1 and BSE	Z97	BSE	BSE	BSE
ARQ/ARQ	SSBP/1 then BSE	Z73	SSBP/1	SSBP/1	SSBP/1
VRQ/ARQ	SSBP/1 and BSE	Z24	SSBP/1	Hybrid	SSBP/1
VRQ/ARQ	SSBP/1 then BSE	Z20	SSBP/1	SSBP/1	SSBP/1
VRQ/VRQ	Natural scrapie then BSE	75x44	Scrapie	Scrapie	Scrapie

a) Genotype of animal at position 136, 154 and 171
b) TSEs that animals were inoculated with
c) Animal identification numbers
d) Incubation period between injection of TSE material and mice displaying clinical signs of disease in the mouse
e) TSE strain was defined by the neuroanatomical distributions of brain lesions
f) Identity of PrP^{Sc} as tested by core (6H4) and N-terminal (P4) primary antibody probing of western blots

Mice strains used in the bioassay were RII, C57BL and VM.

The panel of external assays applied to ARQ/ARQ sheep tissues from animals that received a delayed BSE infection indicate the majority of these animals had a dominant BSE infection (table 6.4). The

Incubation periods of the infections were typical of BSE infections. However in one animal (Z115) BSE was not detected in the brain by western blotting, PrP^{Sc} distribution profile or by complex immunohistochemistry, instead scrapie was the dominant pattern and scrapie-like PrP^{Sc} was present. The sPMCA assay was able to detect BSE in the brain, spleen and prescapular lymph node of this animal. The sPMCA is a highly sensitive assay which has previously been demonstrated to outperform conventional tests in detecting BSE when BSE was present in excess scrapie, therefore low levels of BSE PrP^{Sc} may have been missed by IHC and western blotting (Simmons et al., 2015). The results of animal Z115 show that in some instances of scrapie and BSE co-infections, testing by brain profiling, western blotting and IHC methods would indicate that the dissemination of BSE PrP^{Sc} is affected by the presence of an established scrapie infection and brain samples from some animals would contain only scrapie BSE PrP^{Sc}. While the presence of a primary scrapie infection may have slowed the progression of BSE PrP^{Sc} to the brain and it may not have been the dominant form of PrP^{Sc}, it was still detected by sPMCA. This is in accordance with previous findings the sensitivity of sPMCA is greater than conventional tests, and as a result conventional testing may not diagnose the presence of BSE if excess scrapie is present in an animal (Simmons et al., 2015).

VRQ/ARQ sheep that received both SSBP/1 and BSE on the same day all had incubation periods that were typical of SSBP/1 lasting on average $233^{+/-}17$ days post inoculation, SSBP/1 alone incubation periods were $240^{+/-}30$ days and BSE incubated for >1008 days. PrP^{Sc} detected by western blotting in the brain of all of the sheep tested had a scrapie 6H4 and P4 reactivity. All 6 of these animals were scrapie positive in the brain by IHC. Dissemination of PrP^{Sc} in regions of the brain indicated that the TSE infections were scrapie aside from Z154 which did not stain strongly enough for PrP^{Sc} to enable typing. Brain tissue from animal Z24 was analysed in 3 mouse bioassays and the corresponding incubation period and western blotting data indicated that the TSE present in the sheep was SSBP/1, although the lesion profiles in the mice were not always definitive, displaying a hybrid profile between BSE and scrapie (table 6.5). Transmission to VM mice did not produce lesion profiles which could be analysed and C57BL mice profiles coincided with SSBP/1 alone. However RIII mice showed a pattern indicative of SSBP/1, but also peaked in a brain region affected by BSE, though overall the results were indicative of a SSBP/1 infection. These data correlated with the sPMCA data where no BSE prion was found in any of the 5 sheep in brain or LRS.

The VRQ/ARQ sheep that received a subcutaneous injection of scrapie followed by an injection of BSE 3 months later appeared to be infected purely by SSBP/1. The incubation period of these animals were scrapie like $246^{+/-}39$ days and the western blots from this experimental group reacted strongly with both 6H4 and P4 antibodies. The PrP^{Sc} was distributed in regions of the brain resembling a SSBP/1 infection, and IHC found SSBP/1 only in the brains of all animals. The mouse bioassays conducted using

brain tissue from this group also gave only SSBP/1 infection in incubation periods, lesion profiles and western blotting results. Again, these data correlated with the sPMCA data where no BSE prion was found in any of the 5 sheep in brain or LRS.

Of The flock of VRQ/VRQ genotype sheep that naturally develop scrapie that were subsequently injected with BSE only scrapie was detected in all conventional tests applied; incubation times, western blotting and mice bioassays. This is in contrast to sPMCA when BSE was detected in the brain of one animal (75x44) that was injected with BSE at 8 months of age.

Data from both the sPMCA and conventional assays that were applied to these ovine tissues build a complex picture of mixed infections with many factors contributing to the progression of each disease within one host. The results described here represent the first documented co-infection of scrapie and BSE in an ovine host and demonstrate that both TSEs can replicate in parallel in certain circumstances and be recovered as distinct entities, at least in an experimental setting. To date, mixed TSE infections in the same host have been documented in animals but examples are limited and many questions still remain unanswered. Natural examples of mixed TSE infections are limited to atypical and classical scrapie strains identified in a sheep which was identified after an outbreak of scrapie in a flock that had a mixture of classical and atypical scrapie pathological presentation and PrP^{Sc} molecular characteristics (Mazza et al., 2010). In one animal the IHC brain profile resembled both atypical and classical scrapie dissemination, and both strains appeared to co-exist in the brain. The PrP^{Sc} in the brain of this individual resembled the other 5 cases examined from the flock in terms of glycoform triplet when western blotted, but also contained an 11-12 kDa fragment which is found in atypical scrapie cases when detected with P4 and L42 antibodies. In addition analysis of archived samples from of ~1850 ovine samples dating from the 1960's found 2 cases in which there was evidence of both atypical and scrapie infections present in the same animal (González et al., 2015). Experimental cases of mixed TSE infections include strains of TME that have been isolated in hamster bioassay that had distinct disease and biochemical characteristics named hyper (HY) and drowsy (DR). HY strain is characterised by shorter incubation time, cerebellar ataxia and hyper excitability, whereas DR infected animals have greater incubation times and suffer from progressive lethargy (R. A. Bessen and Marsh, 1992). HY no longer causes disease in mink but the DR strain remains infectious, and the PrP^{Sc} have different susceptibilities to proteases and molecular weights of their respective PrP^{Sc} (Bartz et al., 2000; Bessen and Marsh, 1994). Infection of hamsters with the two isolated strains can lead to inhibition of HY by the DR strain, which is reflected by the *in vitro* amplification in sPMCA where seeding reactions with a mixture of the two at different concentrations led to a reduction in amplification of hyper alone (Shikiya et al., 2010). Co-infection of the two strains prolonged the HY strain incubation period and led to PrP^{Sc} deposition in tissues that reflected DR strain rather than HY

strain. The inhibition displayed by DR and the particular cellular dissemination of the two strains led the researchers to conclude that the most likely explanation of strain interference in this case was competition for PrP^C as a limited cellular resource or other cofactors for replication, which was corroborated by the sPMCA data. Two discrete scrapie strains have also been isolated from the same ovine brain tissue when analysed in mouse bioassay (Thackray et al., 2012). In this experimental passage of prions, there is still doubt as to whether in the original inoculum contained separate TSE entities or whether by introducing an infectious agent to the non-classical host may have caused strain mutation and generated a new strain as well as the original infectious agent.

Considering the BSE and scrapie co-infections investigated here, it is clear that the genotype of the sheep had a large influence on whether or not mixed infections could occur. Unsurprisingly, of the animals tested the ARQ/ARQ sheep that were more susceptible to BSE infections and contained BSE in all tissues in the majority of animals even when administered up to 3 months after scrapie. This was not the case for ARQ/VRQ animals, which are more resistant to BSE infections, even animals that received BSE and scrapie on the same day showed no signs of a BSE infection. This information may prove to be valuable in directing surveillance testing for BSE co-infections towards animals most at risk of BSE infections to aid identification of mixed infections in the field. Furthermore the data demonstrates that at least in ARQ/ARQ and VRQ/VRQ genotypes of sheep when co-infected with scrapie and BSE mixed infections are possible and dissemination of both BSE and scrapie occurs in a variety of tissues, including the brain, spleen and prescapular lymph node. Both BSE and scrapie have the potential to be widely disseminated in tissues, and the dominant PrP^{Sc} found in the brain by conventional testing may not be the only infectious PrP^{Sc} found throughout the animal.

As BSE is zoonotic and scrapie has never been documented to cause disease in humans after being known for over 250 years, the dissemination of the PrP^{Sc} throughout the tissues of animals that could potentially enter the human food chain is of great interest. When both TSEs were administered at the same time to permissive animals there was widespread distribution of BSE prion in the different tissues tested by sPMCA. BSE was found in the brain of all animals (5/5) in the spleen of 80% (4/5) and the prescapular lymph node of 60% (3/5). When scrapie had time to establish in an animal before BSE was administered the tissue distribution of BSE dropped, it was found in 60% of brains (3/5) 60% of spleens (3/5) and 60% of prescapular lymph nodes (3/5). In this group of animals, tissue distribution showed the greatest variance, some contained BSE in the brain but none in the peripheral tissues, whereas for some individuals the converse was true. In cattle BSE it has previously been shown to distribute in many of the central nervous system tissues and throughout peripheral tissues including spleen, peyers patches, optical nerves, lymph nodes and rumen when assayed using sPMCA (Franz et al., 2012). Although when using classical immunohistochemistry to detect BSE PrP^{Sc} dissemination in

cattle even at end point disease appears limited to the central nervous system, including the enteric nervous systems and gut associate lymphatic system such as Peyer's patches with very little peripheral dissemination (Hoffmann et al., 2011, 2007; Kaatz et al., 2012; Terry et al., 2003). In contrasts BSE transmission to ARQ/ARQ sheep has demonstrated an accumulation of PrP^{Sc} in a wide range of tissues by immunohistochemitry, including the central nervous system, the peripheral nervous system, lymphorecticular system, peyers patches, spleen and prescapular lymph node (Bellworthy et al., 2005; Jeffrey et al., 2001). The distribution of scrapie PrP^{Sc} in sheep involves the central nervous system and the peripheral nervous system, with lymphorecticular distribution such as the spleen, mesenteric and prescapular lymph node, peyers patches and the kidneys (Heggebø et al., 2002; Sisó et al., 2006; van Keulen et al., 1996).

Previously in this thesis (chapter 5; (Simmons et al., 2015)) the sPMCA assay has been compared to statutory tests currently recommended by the European Union reference laboratory. In this comparison the sPMCA was far more sensitive at detecting BSE prions in mixtures containing greater amounts of 3 classical and 1 atypical scrapie isolates than current statutory tests of ELISA, western blotting and animal bioassay. The sPMCA was able to correctly identify 0.1% BSE when mixed with each classical scrapie strain and 0.01% in atypical scrapie, with 100% specificity and sensitivity. Here, when analysing *in vivo* co-infection, BSE was detected in the tissues of two animals via sPMCA that conventional testing ruled as scrapie. BSE was not found in the brain of Z115 (ARQ/ARQ) by western blotting, lesion profiling or IHC, yet this tested positive by sPMCA. A sheep with a natural scrapie infection that received BSE at 8 months also contained BSE detected by sPMCA but this was not picked up by the panel of conventional tests, including the current gold standard bioassay that is recommended by the European Union reference laboratory. These results may indicate that the sensitivity of conventional testing may not be as sensitive as sPMCA and therefore may miss potential mixed infections, in accordance with earlier findings. This is particularly true regarding the natural scrapie infection as this model may not be comparable to other groups in terms of experimental control but is the closest to a natural mixed infection as is currently known. Widespread BSE transmission to cattle is thought to have taken place through BSE infected MBM feedstuffs. Small ruminants were also fed MBM during the time that BSE would have been circulating within the product, and while they can be experimentally infected with BSE via the oral route no natural cases of BSE in sheep have been documented and only two in goats despite intensive surveillance (Eloit et al., 2005; Foster et al., 1993; Jeffrey et al., 2006b; Stack et al., 2006). The absence of BSE detected in small ruminants may be due to the sensitivity of conventional tests when present in mixed infections, as scrapie was also endemic in the UK sheep flock. It is conceivable that mixed TSE infections containing BSE in small ruminants may have been miss diagnosed as scrapie.

In ARQ/ARQ animals, data indicate that co-infections with BSE and scrapie had reduced dissemination of BSE in peripheral tissues tested by sPMCA than those that received only BSE. Animals that received delayed BSE inoculation were also less likely to have sPMCA-detectable BSE in the brain, and one animal did not have BSE in any tissue tested. Therefore overall, the presence of scrapie appeared to have an inhibitory role on the disease progression of BSE. Iatrogenic CJD PrP^{Sc} replication in PMCA has been shown to be inhibited by truncated recombinant human PrP^{C} which may act as a competitive inhibitor and several ovine recombinant PrP peptides have also inhibited conversion of PrP^{C} to PrP^{Sc} in sPMCA (Rigter et al., 2009; Yuan et al., 2013). Furthermore in sPMCA reactions, hamster adapted TME strains have displayed strain inhibition (Shikiya et al., 2010). Reactions seeded with hyper strain were shown to be inhibited by the addition of drowsy strain PrP^{Sc}. In mixed infections scrapie and BSE PrP^{Sc} may compete for PrP^{C} *in vivo* as the protein misfolding substrate, therefore the presence of scrapie in an animal will reduce the available PrP^{C} for BSE and inhibit BSE replication in that host.

Application of sPMCA and other conventional assays to sheep that received both BSE and scrapie have demonstrated that both TSEs can replicate in mixed infections in the same ovine host. The largest determinant as to whether these mixed infections can occur appears to be permissibility for both TSEs, which is dictated by the genotype of the animal for PrP^{C}. The most 'at risk' genotype tested for co-infection where BSE propagates was the ARQ/ARQ animals, 90% of animal tested by sPMCA had BSE present (9/10). Dissemination of BSE in the periphery seems to be complex and more in depth studies would be needed to ascertain if scrapie has any effect on this process. However these co-infected animals have proved that the dominant TSE in the brain may not necessarily match that in the periphery. In addition, this data shows that it is possible in some ovine co-infections BSE may be missed by a panel of current testing methods, and can only be detected by sPMCA, however it is currently unknown whether this low level of PrP^{Sc} could pose a zoonotic threat.

Chapter 7. Identification and characterisation of bovine BSE which upon *in vitro* amplification demonstrates biochemical characteristics of scrapie in a genotype dependent manner

7.1. Introduction

Transmissible spongiform encephalopathies are defined by the presence of a misfolded conformer of the protein PrP^{C}, known as PrP^{Sc} (Prusiner, 1982). While this misfolded conformer is the common and causative factor in TSEs, there is variation in the specific conformation of PrP^{Sc} between TSEs and TSE strains. These differences in three dimensional structure between PrP^{Sc} from different diseases or strains can be used to identify novel prions as they have distinct biochemical characteristics. Such traits have also been routinely used to differentiate known prion strains such as scrapie and BSE (Hope et al., 1999).

BSE was first described in 1986 and went on to cause an epidemic in the UK in which 180,000 cattle were confirmed to be infected, and estimates of cattle exposure to the BSE agent range from 1 to 3 million individuals (Smith et al. 2003). While BSE primarily affects cattle, BSE can be transmitted to a range of species and is thought to be responsible for the human variant CJD infections that developed in the UK since the early 1990s as a results of consuming infected beef (Will et al., 1996). Furthermore, sheep can be experimentally susceptible to BSE with a range of different *PRNP* genotypes at amino acid positions 136, 154 and 171 in which the variations of PrP^{C} genotype result in different permissibility to misfolding by PrP^{Sc} (Goldmann et al., 1991; Houston et al., 2003).

During the BSE epidemic there was concern that the BSE agent could also be spread to the UK sheep population which likely consumed the same BSE-contaminated feed as cattle. Because the clinical disease presentation for BSE is very similar to scrapie, biochemical and immunohistochemical tests were developed to differentiate the PrP^{Sc} from these prion strains and the tests were introduced to routine surveillance to screen for any BSE present in the UK and European sheep flocks (Gretzschel et al., 2005).

A number of biochemical assays can be applied to differentiate between BSE and scrapie, primarily based on differential cleavage sites for proteases in the N-terminal region of PrP^{Sc} and subsequent reactivity with N-terminal antibodies. Scrapie isolates (aside from CH1641) contain protease-resistant PrP^{Sc} with an N-terminal domain that is not present in BSE PrP^{Sc}, therefore antibodies that recognise this region, such as monoclonal antibody P4 (figure 2.1), can be used to distinguish between PrP^{Sc} from scrapie and BSE after PK digestion and western blotting (Stack et al., 2002). Furthermore, different conformations of PrP^{Sc} favour different glycotypes of PrP^{C} for misfolding, BSE contains roughly equal proportions for both the di-glycosylated and mono-glycosylated protein, but scrapie has a preference for the di-glycosylated form, typically containing double the amount of mono-glycosylated PrP^{Sc} (Stack et al., 2002). Scrapie and BSE conformations of PrP^{Sc} also display differences in stability when treated

with chaotropic agents such as guanidine hydrochloride, and the extent of this denaturation of PrP^{Sc} can be used to biochemically distinguish PrP^{Sc} of scrapie and BSE (Peretz et al., 2001; Pirisinu et al., 2011).

Within the duration of the study described in this thesis, a phenomenon has been described in which the *in vitro* amplification of ovine and bovine BSE in ovine VRQ/VRQ substrate (for positions 136, 154 and 171) leads to the formation of PrP^{Sc} with biochemical characteristics of scrapie, even though derived from BSE (Krejciova et al. 2014). In parallel with this published research, the present study also observed this switch in PrP^{Sc} molecular phenotype upon sPMCA amplification of bovine BSE in ovine VRQ substrate. As amplification of prions in sPMCA can be used as a model for the *in vivo* evolution PrP^{Sc} (Boerner et al., 2013) the present study sought to 1) determine if this was an inherent quality of BSE, and if not all BSE isolates could change molecular phenotype how common was this ability within bovine BSE isolates, 2) to fully characterise any PrP^{Sc} generated that had undergone a molecular switch using a range of assays, and 3) to assess if any BSE prions were still present in the scrapie-like PrP^{Sc} derived from bovine BSE.

7.2. Results

7.2.1. Identification of BSE with molecular plasticity traits.

Bovine BSE sPMCA products have been generated with a molecular weight more typical of scrapie from seeds with BSE molecular weights on SDS-PAGE western blots (Krejciova et al. 2014). The change in the molecular weights of the sPMCA products appeared to be dependent on the genotype of the ovine substrate with only VRQ/VRQ (at amino acid positions 136, 154 and 171) substrate facilitating the abnormal sPMCA products.

In parallel to this research, the current study analysed a panel of 5 bovine BSE isolates amplified for 5 rounds in bovine and ovine VRQ/VRQ, ARQ/ARQ and AHQ/AHQ substrates (figure 7.1). All isolates demonstrated some amplification in bovine, AHQ/AHQ and VRQ/VRQ substrates but not in ARQ substrate. One of the samples (SE1929/0749) appeared to produce a sPMCA product in VRQ/VRQ with a molecular weight typical to that of scrapie (figure 7.1A). Amplification of SE1762/0013 (figure 7.1D) and SE1946/0003 (figure 7.1E) in VRQ/VRQ substrate produced protease-resistant PrP^{Sc} with molecular weight typical of bovine BSE while the amplification of SE1945/0035 and SE1762/0007 were too weak to interpret (figure 7.1B and 7.1C respectively). All bovine BSE samples amplified in bovine and ovine AHQ/AHQ substrates produced protease-resistant PrP^{Sc} with molecular weights typical of BSE.

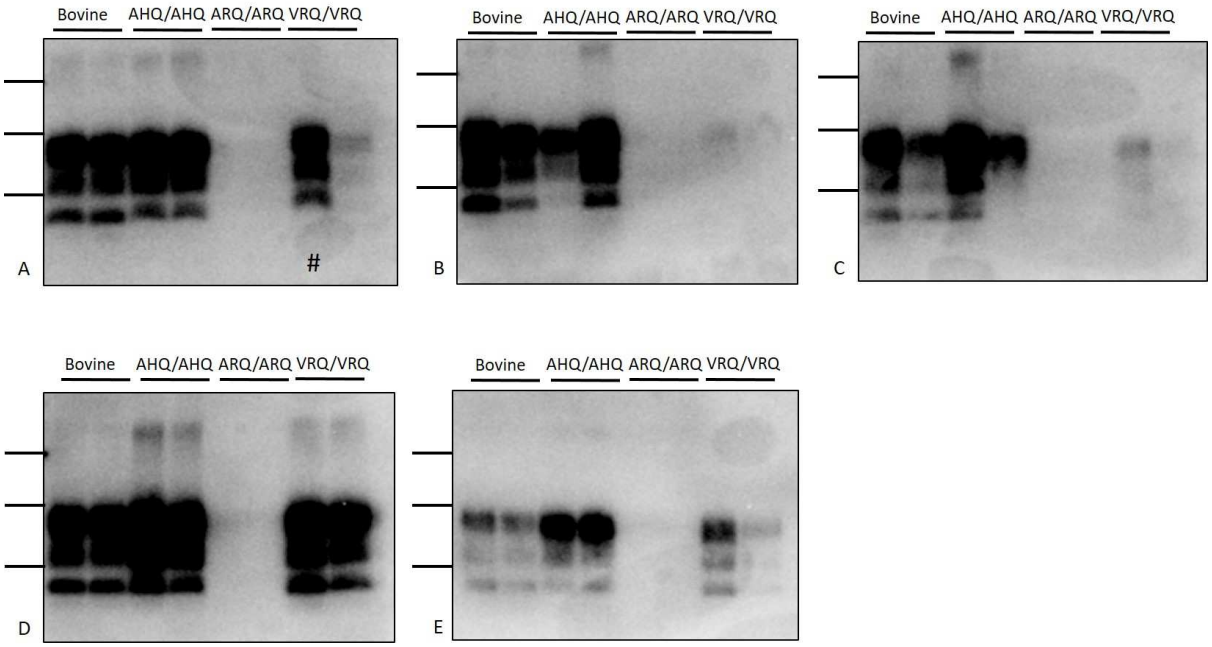


Figure 7.1. Amplification of bovine BSE in bovine and ovine substrates. Bovine BSE isolates, SE1929/0749 (A), SE1945/0035 (B), SE1762/0007 (C), SE1762/0013 (D), and SE1946/0003 (E) were amplified for 5 rounds in bovine and ovine AHQ/AHQ, ARQ/ARQ and VRQ/VRQ substrates as indicated. High molecular weight product on blot A indicated with #. Following amplification sPMCA products were digested with 50 µg/ml PK, separated in SDS-PAGE, before separated proteins were transferred to PVDF membrane and probed with SHa31 primary antibody. Molecular weight markers are indicated at 40, 30 and 20 kDa.

Following the identification of a bovine BSE sample which demonstrated that sPMCA products with distinct molecular weights could be generated depending on the *PRNP* genotype of the substrate, and the low amplification efficiency (1/2 replicates) of these products, a larger cohort of BSE samples (table 7.1) were amplified in bovine, ovine AHQ/AHQ and VRQ/VRQ substrates with higher numbers of replicates (representative blots displayed in figure 7.2).

Table 7.1. Bovine BSE 10%* (w/v) amplified in ovine VRQ/VRQ and AHQ/AHQ substrates

Sample ID[a]	Details[b]	AHQ/AHQ[c]	VRQ/VRQ[d]	Bovine[e]
FT5026/0037	Brain pool of 4 BSE isolates (PG1197/92. PG1840/92, PG1842/92 and PG1880/92)	5/5	5/5	2/2
SE0253/0055	One isolate of BSE (PG0475/05)	5/5	1/5	2/2
Brain Pool 1 (BP-1)	Brain pool of 10 20% (w/v) BSE homogenates (PG0091/04, PG00034/04, PG01184/02, PG01257/02, PG1412/02, PG00354/03, PG000365/03, PG00449/03, PG00487/03, PG00572/03)	5/5	5/5	2/2
SE1762/0013	Brain pool of 4 BSE isolates (RBSE 97/00935, RBSE 97/00982, RBSE 97/01002 and RBSE 97/01133)	5/5	5/5	2 /2
SE1945/0035	Brain pool of 71 BSE isolates	5/5	0/5	2/2
SE1762/0007	Brain pool of 4 BSE isolates (RBSE 97/04810, RBSE 97/04001, RBSE 98/00948 and RBSE 98/01764)	5/5	1/5	2/2
Brain Pool 2 (BP-2)	10% Brain pool of unknown BSE positive isolates (YLT1408)	5/5	2/5	1/2
SE1929/0749	1% (w/v) Brain pool of 71 BSE isolates	10/10	4/10	2/4

a) Sample identification for BSE isolates and pools
b) Details relating to composition of the sample
c) Number of positive reactions when amplified in ovine AHQ/AHQ substrate
d) Number of positive reactions when amplified in ovine VRQ/VRQ substrate
e) Number of positive reactions when amplified in bovine substrate

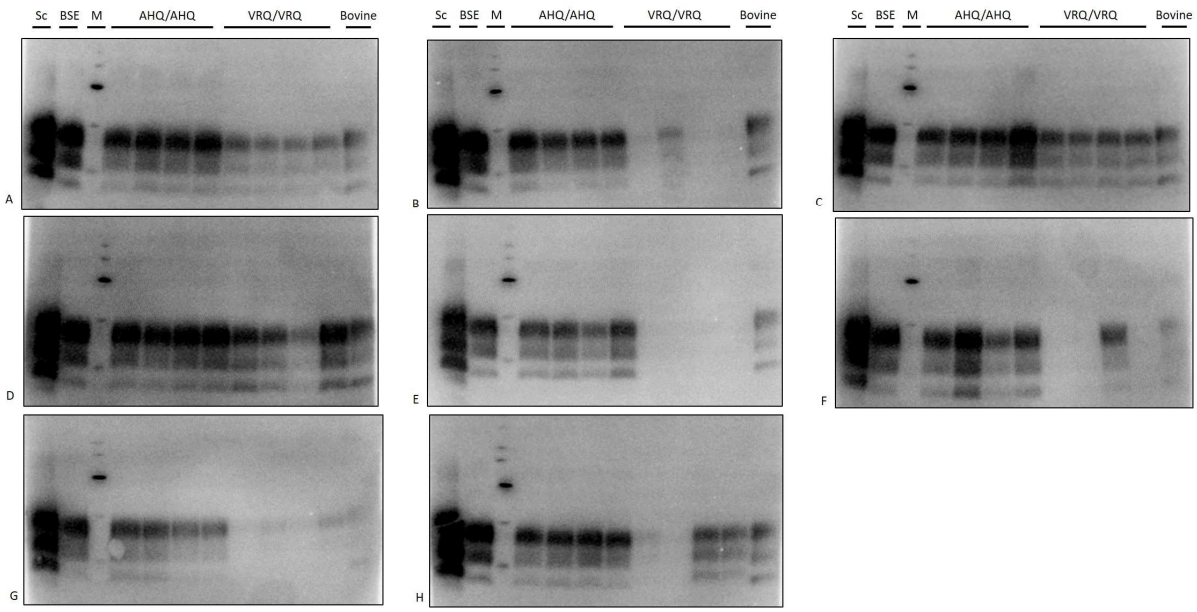


Figure 7.2. Amplifications of bovine BSE samples in ovine and bovine substrates. Bovine BSE isolates were amplified for 5 rounds in ovine AHQ/AHQ, VRQ/VRQ or bovine substrates (as indicated) and the Pk-resistant products analysed on western blots probed with SHa31 antibody. The samples shown are scrapie blotting control (Sc), BSE blotting control (BSE), molecular weight markers (M), sPMCA products amplified in AHQ/AHQ substrate (x4), VRQ/VRQ substrate (x4) and bovine substrate (x1). The BSE used as spikes were FT5026/0037 (A), SE0253/0055 (B), BP-1 (C), SE1762/0013 (D), SE1945/0035 (E), SE1762/0007 (F), BP-2 (G), SE1929/0749 (H). The blots are representative of data shown in table 1.

All BSE samples displayed the highest amplification efficiency in AHQ/AHQ substrates, all replicates from all samples amplifying efficiently. All replicates of FT5026/37, BP-1 and SE1762/0013 amplified in VRQ/VRQ substrate, however amplification of the other BSE samples was less efficient. 40% of BP-2 and SE1929/0749 reactions showed detectable products after 5 rounds of amplification (2/5 and 4/10 respectively), while 20% (1/5) of SE0253/0055 and SE176/0013 were positive. SE1935/0035 failed to amplify at all in VRQ/VRQ substrate. All samples that amplified demonstrated 100% amplification efficiency in bovine substrate apart from BP-2 and SE1929/0749 which both only demonstrated PK resistant sPMCA products in 50% of replicates.

sPMCA products with unusually high molecular weights for BSE were only found in SE1929/0749 replicates when amplified in VRQ/VRQ substrate. 10 sPMCA products from SE1929/0749 amplified in AHQ/AHQ substrate were all typical of classical BSE in terms of molecular weight.

7.2.2. Isolation of multiple populations of BSE PrP^{Sc} with distinct molecular phenotypes.

Following the initial amplification of BSE with high molecular weight, further amplification of SE1929/0749 was carried out in both AHQ/AHQ and VRQ/VRQ substrates. To characterise the amplifications further, glycosylation profiling was used. The relative percentage of the di-glycosylated, mono-glycosylated and un-glycosylated bands are specific for scrapie and BSE and have previously been described to differentiate the two strains (Vulin et al., 2011). A total of 60 replicates of SE1929/0749 were amplified in VRQ/VRQ substrates on numerous occasions, of these, 40 samples gave PrP^{Sc} western blot signals suitable for glycoform profiling. The percentage of di-glycosylated band was plotted against the mono-glycosylated band as measured via densitometry of the western blots and were compared to data generated with scrapie and other BSE isolates amplified under the same conditions (figure 7.3A). The controls consisted of 5 replicates clustered for each of the scrapie and BSE samples; scrapie was characterised by between 44-51% di-glycosylated and 30-33% mono-glycosylated bands and BSE control di-glycosylated content ranged from 62-65% and 24-25% mono-glycosylated protein. The profile of SE1929/0749 showed far greater variability in the ratio of di-glycosylated protein to mono-glycosylated isoforms, with individual amplifications ranging from even higher diglycan content than that characteristic of BSE to even higher monoglycan content compared to that characteristic of scrapie. In comparison, amplification of 33 replicates of SE1929/0749 in AHQ/AHQ substrate was far more consistent (figure 7.3B) and all of the replicates clustered around the ratios produced with BSE controls. To facilitate further investigation the SE1929/0749 sPMCA products were split into 3 populations based on the glycosylation profiles, which were amplifications with a BSE-like profile ($BSE_{1:1}$), a scrapie-like profile ($Sc_{1:1}$) and an intermediate profile ($Int_{1:1}$) (figure 7.3A). Two replicates of each of these populations along with the two replicates of AHQ/AHQ amplified SE1929/0749 (denoted as $BSE_{A1:1}$) (circled in figure 7.3A and 7.3B) were further characterised by discriminatory antibody western blotting.

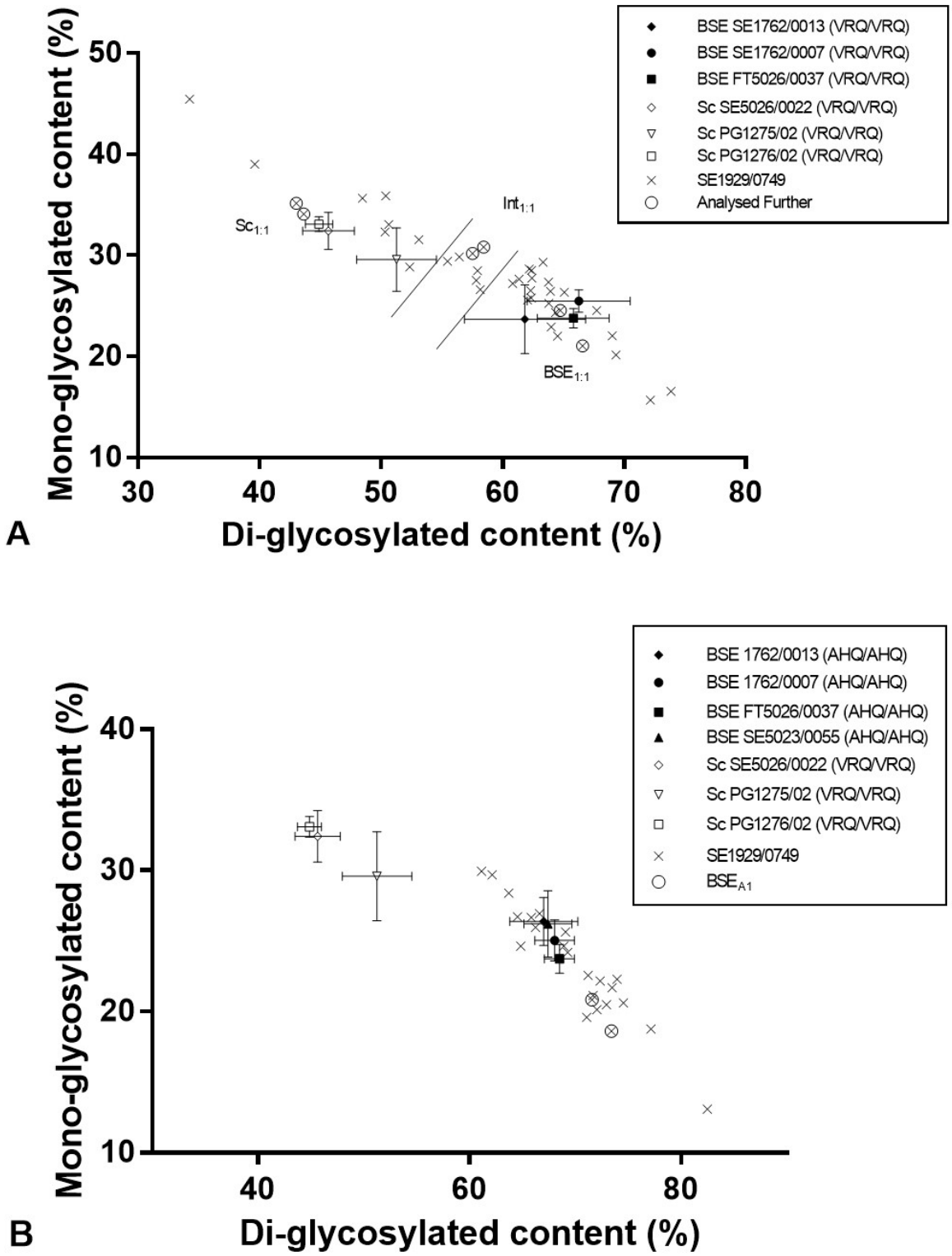


Figure 7.3. Glycosylation profiles of sPMCA products. A) BSE sample SE1929/0749 amplified in ovine VRQ/VRQ substrate. The relative abundance of di-glycosylated and mono-glycosylated protein bands as measured by densitometry of western blotted round 5 sPMCA products of SE1929/0749 and scrapie and BSE controls. Each product of sPMCA for SE1929/0749 amplification is

represented by x, those selected for further analysis are circled. Non-filled symbols with standard deviations are scrapie sPMCA amplified controls; square - PG1276/02, triangle - PG1275/02, diamond - SE5026/0022. Filled symbols with standard deviations depict BSE sPMCA amplified controls; square - FT5026/0037, circle - SE1762/0007 and diamond - SE1762/0013. Three distinct populations are characterised base on these glycoform profiles, identification of which is aided by the use of the two separator lines. To the left of both lines closest to the scrapie controls lies a population designated scrapie-like or $Sc_{1:1}$. Between the two lines are the intermediate profiles or $Int_{1:1}$ and to the right of both lines and clustered around the BSE controls are the BSE-like population or $BSE_{1:1}$**. B) Glycosylation profiles of BSE sample SE1929/0749 amplified in ovine AHQ/AHQ substrate.** SE1929/0749 samples are depicted by x and those selected for further analysis are circled and are characterised as $BSE_{A1:1}$. Scrapie sPMCA amplified controls are represented by non-filled symbols with standard deviations; square - PG1276/02, triangle - PG1275/02, diamond - SE5026/0022. Filled symbols with standard deviations depict BSE sPMCA amplified controls; square – FT5026/0037, circle SE1762/0007, diamond - SE1762/0013 and triangle - SE5023/0055.

7.2.3. Probing glycoform populations with discriminatory antibodies.

BSE (of both bovine and ovine origin) and scrapie PrP^{Sc} can be differentiated by their relative reactivity to core (SHa31) and N-terminal antibodies (P4), scrapie PrP^{Sc} reacts strongly with both antibodies whereas the P4 epitope is mostly lost in BSE (Gough et al., 2014). As noted, amplification of SE1929/0749 yields relatively high molecular weight PK-resistant products, and a spectrum of different glycosylation profiles. The three populations identified in VRQ/VRQ amplification (figure 7.3A) and the sPMCA products of AHQ/AHQ amplifications (figure 7.3B) were probed with both SHa31 and P4 to assess if a relationship between glycoform profile and antibody reactivity could be found. The sPMCA products that were tested by this method are circled in figures 7.3A and 7.3B. PrP^{Sc} from the $BSE_{A1:1}$ population gave strong SHa31 signal and though signal from the $BSE_{1:1}$ population was weaker, neither gave strong signals with P4 (figure 7.4A and 7.4B respectively) which is typical of BSE. However $Sc_{1:1}$ and $Int_{1:1}$ populations gave positive results with both antibodies (figure 7.4A and 7.4B), strong reactivity with both of these antibodies is not known to occur with PrP^{Sc} from classical BSE (ovine or bovine) but is characteristic of scrapie PrP^{Sc}. Densitometric methods previously used in chapter 5 and published material (Gough et al., 2014; Simmons et al., 2015) were employed to interpret the signal ratio between SHa31 and P4 antibodies (table 7.2). The densitometry data of SE1929/0749 in AHQ/AHQ substrate ($BSE_{A1:1}$) and VRQ/VRQ substrate ($BSE_{1:1}$) with a BSE glycoform profile also displayed reactivity with SHa31 and P4 that is characteristic of BSE. However, densitometric analysis of the $Sc_{1:1}$ and $Int_{1:1}$ populations resulted in a SHa31:P4 ratio that would indicate the PrP^{Sc} is of scrapie origin or not typically BSE-like.

A summary of the glycosylation profiles and antibody reactivity of the four different populations analysed can be found in the primary PMCA (top panel) summary (figure 7.8). The sPMCA products that are circled in figure 7.3 represent the primary PMCA were used to seed a secondary sPMCA.

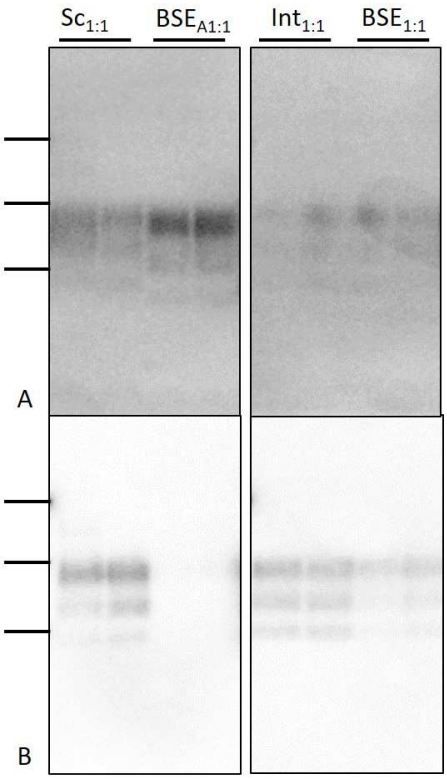


Figure 7.4. SE1929/0749 sPMCA products probed with SHa31 and P4 antibodies. Populations of sPMCA amplified SE1929/0749 consisted of $Sc_{1:1}$, $BSE_{A1:1}$, $Int_{1:1}$ and $BSE_{1:1}$ were identified by characteristic glycoform profiles. These populations were further digested, SDS-PAGE electrophoresed, western blotted and probed with SHa31 (**A**) and P4 (**B**) primary antibodies. Molecular markers are indicated at 40, 30 and 20 kDa.

Table 7.2. Densitometry of primary passage SE1929/0749 populations.

Sample[a]	SHa31[b]	Positive[c]	P4[d]	Ratio[e]	Normalise[f]	Verdict[g]
$Sc_{1:1}$	13049.79	Yes	8843.79	1.48	0.47	Scrapie
$Sc_{1:1}$	12759.08	Yes	11810.86	1.08	0.34	Scrapie
$BSE_{A1:1}$	19229.61	Yes	344.68	55.79	17.78	BSE
$BSE_{A1:1}$	21681.14	Yes	473.92	45.75	14.58	BSE
$Int_{1:1}$	8830.664	No	N/A	N/A	N/A	N/A
$Int_{1:1}$	12142.76	Yes	9800.52	1.24	2.12	Scrapie
$BSE_{1:1}$	13233.95	Yes	4487.71	2.95	5.04	BSE
$BSE_{1:1}$	10440.71	No	N/A	N/A	N/A	N/A

a) Specific population of SE1929/0749 analysed by SHa31 and P4 discriminatory western blotting
b) Signal of sample when probed with SHa31 primary antibody
c) Positivity of sPMCA reaction. To remove subjective interpretation for a sPMCA product to be considered positive it must exceed a previously generated background value for western blotting (10727.78). "Yes" - indicates threshold value met and replicate is positive, "no" - value not met, as such reaction is not positive and subsequent analysis not applied to that sample.
d) Signal of sPMCA products when probed with P4 primary antibody.
e) Ratio of SHa31:P4 signal
f) Normalisation of ratio by expression as an internal scrapie blotting control (not shown)
g) Normalised ratio indicative of scrapie or BSE (Gough et al., 2014; Simmons et al., 2015). Values lower than 2.9 considered scrapie, greater than 2.9 BSE.

N/A – Reaction not positive (c) therefore analysis not applied.

7.2.4. Secondary sPMCA amplification of the four identified SE1929/0749 populations

The sPMCA amplification in figure 7.4 is analogous to an *in vivo* primary passage, as PrP^{Sc} acclimatises to *in vitro* replication in each substrate and will be subsequently referred to as primary PMCA. An initial spike of 100 µg of brain tissue was diluted throughout sPMCA and digestion until 60 ng of original brain material was analysed in SDS-PAGE electrophoresis, the sensitivity of western blotting in our hands is ~10-30 µg of brain material (KC Gough, personal communication). Therefore it is very unlikely that the seed produces any positive signal in the western blots. To assess how the different populations behave on further amplification in both ovine VRQ/VRQ and AHQ/AHQ substrates, 10 µl of sPMCA products were used to seed sPMCA reactions for 5 rounds in both substrates, described as a secondary PMCA. The secondary PMCA products were subjected to the same glycoform profiling, and characterised by the affinity to core and N-terminal antibodies as products from the initial sPMCA

reactions. In addition, products were also subjected to amplification in alternating substrate sPMCA as described in chapter 5 to ascertain whether any 'BSE prion' is present in the products.

7.2.5. Relative abundance of glycosylated proteins in secondary sPMCA products

Two replicates of the four isolated glycoform populations of SE19292/0749 were further amplified in a secondary PMCA in both ovine AHQ/AHQ and VRQ/VRQ substrates. BSE sPMCA products with a glycosylation profile representative of scrapie isolates ($Sc_{1:1}$) were used to seed sPMCA reactions in VRQ/VRQ substrate for a further 5 rounds of amplification and produced two further distinct populations (figure 7.5A); 50% (3/6) retained a scrapie glycoform profile ($Sc_{2:1}$) while the remaining 50% produced an intermediate glycoform profile ($Int_{2:1}$). The same $Sc_{1:1}$ sPMCA products when amplified in AHQ/AHQ substrate only produced an intermediate phenotype ($Int_{2:2}$) (2/2; figure 7.5B). The initial primary PMCA intermediate glycoform profile ($Int_{1:1}$) when amplified in VRQ/VRQ for a secondary passage was shifted to resemble scrapie in terms of glycoform ($Sc_{2:2}$) (figure 7.5C), yet this shift was not observed in an AHQ/AHQ secondary sPMCA, and the sPMCA products retained the intermediate profile ($Int_{2:3}$) (figure 7.5D). SE1929/0749 samples with a primary PMCA BSE glycoform profile when amplified in VRQ/VRQ ($BSE_{1:1}$), on secondary PMCA in VRQ/VRQ produced glycoform profiles that resembled scrapie ($Sc_{2:3}$) (figure 7.5E) however when amplified in AHQ/AHQ the profiles were characteristic of BSE ($BSE_{2:1}$) (figure 7.5F). sPMCA products originally amplified in AHQ/AHQ substrate produced only BSE glycoform profiles, in further amplifications in either VRQ/VRQ ($BSE_{A2:1}$) (figure 7.5G) or AHQ/AHQ ($BSE_{A2:2}$) (figure 7.5H) substrates. To summarise, primary PMCA populations of $Sc_{1:1}$ and $Int_{1:1}$ had potential to form products with either scrapie or intermediate glycoform profiles in the secondary PMCA and this was dependent on the genotype used for amplification, but they did not produce classical BSE glycoform profiles. $BSE_{1:1}$ could seed secondary PMCAs with products that were representative of scrapie or BSE but the phenotype was dependent on the genotype in which amplification occurred. In contrast initial BSE glycoforms typical of BSE generated in AHQ/AHQ ($BSE_{A1:1}$) substrate could only seed reactions that gave characteristically classical BSE glycoform profiles in either genotype used for amplification.

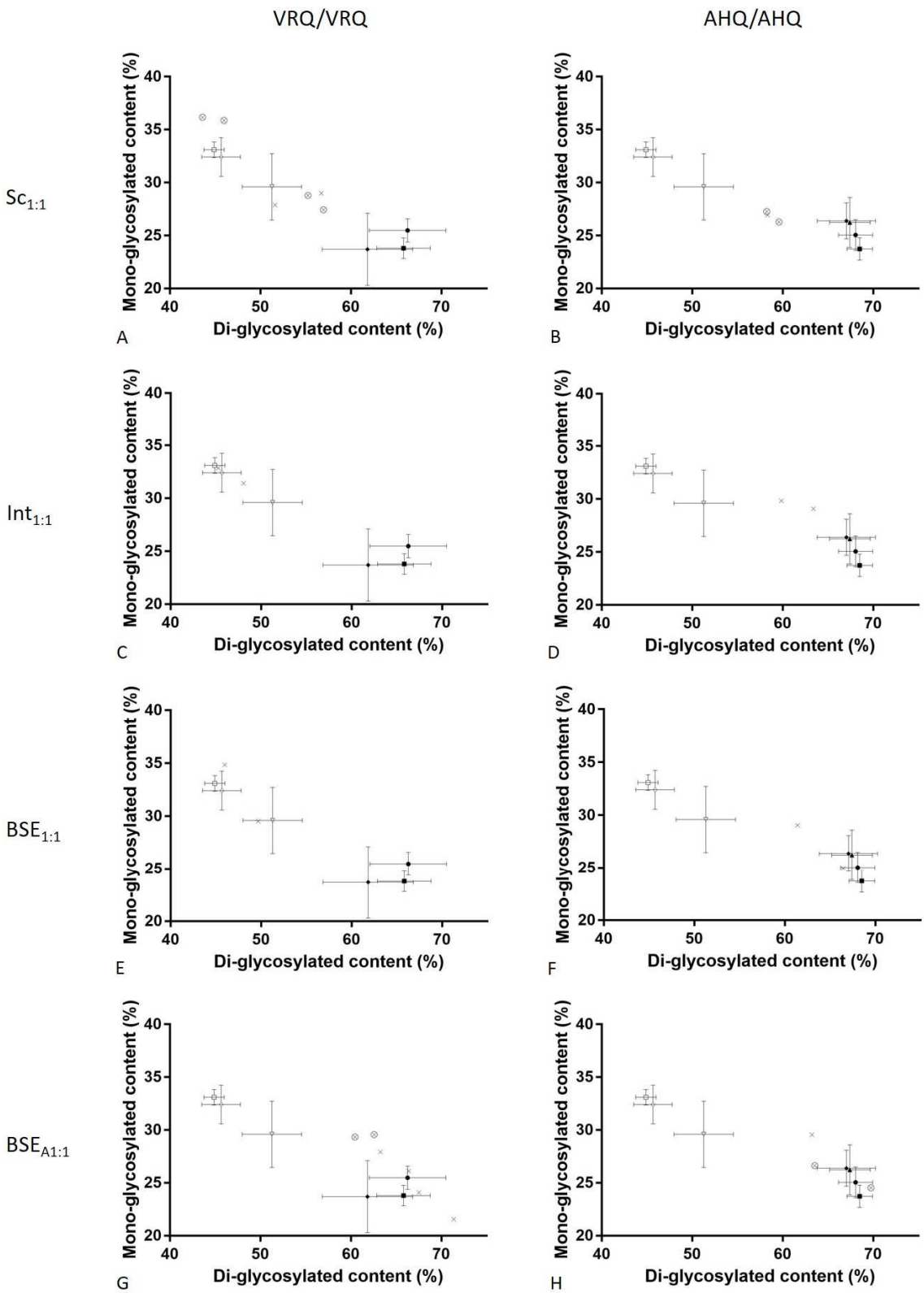


Figure 7.5. Glycoform profiles of secondary passaged SE1929/0749. The four distinct populations of SE1929/0749 that were produced in PMCA in ovine VRQ/VRQ and AHQ/AHQ were used to seed five more rounds of BSE in both VRQ/VRQ (**A**, **C**, **E**, **G**) and AHQ/AHQ (**B**, **D**, **F**, **H**) substrates. The seeds were the primary sPMCA products amplified in VRQ/VRQ with scrapie-like (**A** and **B**) intermediate (**C** and **D**) and BSE-like (**E** and **F**) glycoform profiles and PMCA products with BSE-like glycoform profiles that were amplified in AHQ/AHQ substrate (**G** and **H**). The primary sPMCA

products are indicated to the left of each row. After 5 rounds of secondary sPMCA the products were western blotted, probed with SHa31 and the percentage of di-glycosylated and mono-glycosylated bands measured by densitometry and plotted against each other. The isolates that were selected for further analysis are circled if there were more than 2 replicates for a population.
For VRQ/VRQ graphs, non-filled symbols with standard deviations are scrapie sPMCA amplified controls; square - PG1276/02, triangle - PG1275/02, diamond - SE5026/0022. Filled symbols with standard deviations depict BSE sPMCA amplified controls; square - FT5026/0037, circle - SE1762/0007 and diamond - SE1762/0013.
For AHQ/AHQ graphs, scrapie sPMCA amplified controls are represented by non-filled symbols with standard deviations; square - PG1276/02, triangle - PG1275/02, diamond - SE5026/0022. Filled symbols with standard deviations depicted BSE sPMCA amplified controls; square – FT5026/0037, circle SE1762/0007, diamond - SE1762/0013 and triangle - SE5023/0055.

7.2.6. Antibody reactivity of the secondary PMCA BSE populations

The reactivity of the secondary sPMCA products with core antibody (SHa31) and N-terminal antibody (P4) was measured to determine if the PrP^{Sc} from each population was representative of BSE or scrapie (figure 7.6). The western blots were analysed using ImageJ and the ratios of signal for each antibody expressed as a proportion of an internal scrapie control (table 7.3). The ratios were then compared to a previously generated standard cut off (three standard deviations from the mean of known scrapie SHa31:P4 ratios) and described as typical of scrapie or BSE PrP^{Sc}.

All populations that were generated in VRQ/VRQ with a scrapie like glycoform profile ($Sc_{2:1}$, $Sc_{2:2}$ and $Sc_{2:3}$) reacted with both SHa31 and P4 primary antibodies. The remaining BSE and intermediate glycoform profiles did not react with P4 and the densitometry ratio was typical of pure BSE isolates.

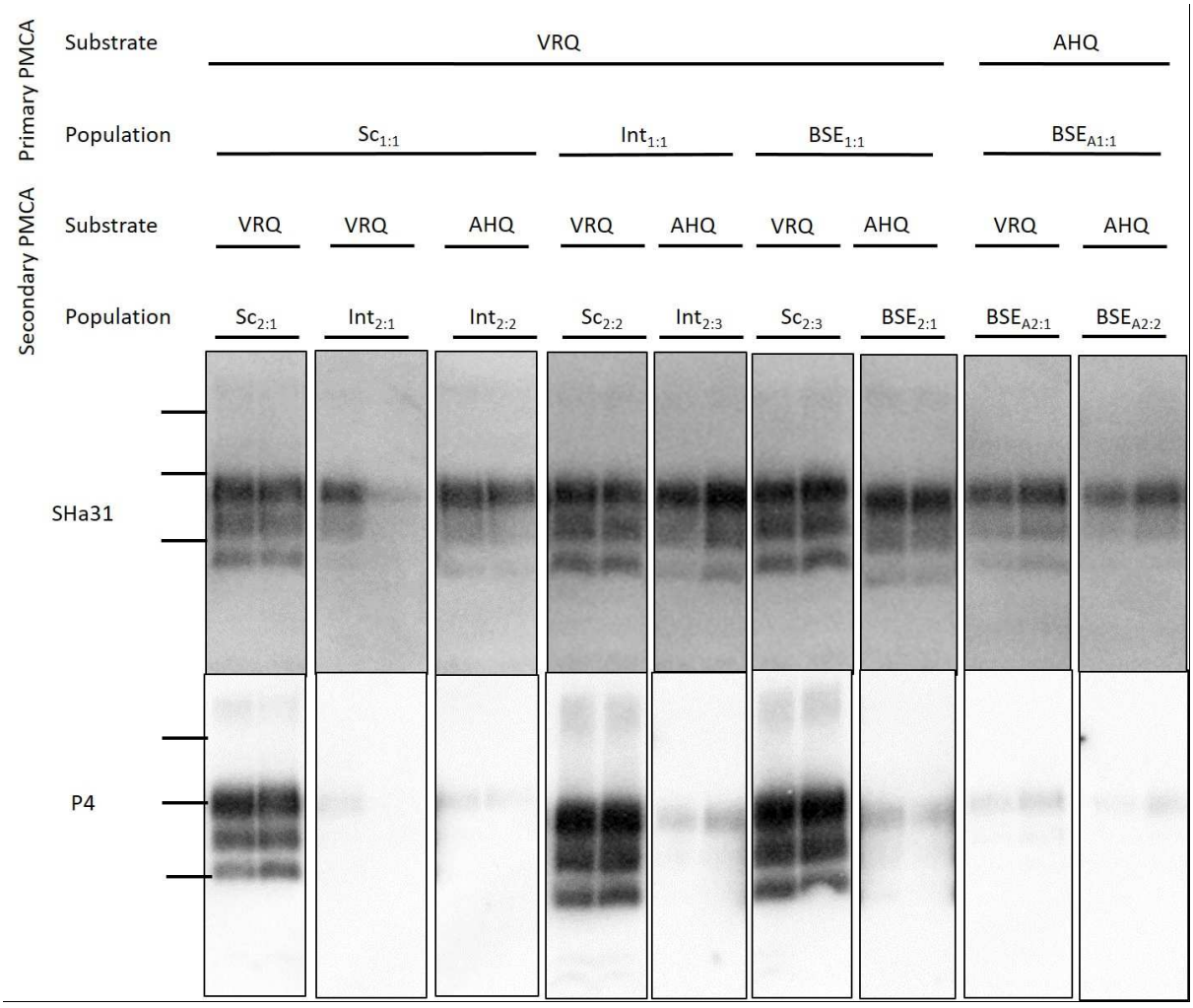


Figure 7.6. SHa31 and P4 reactivity of the secondary PMCA products. The subpopulations of PrP^{Sc} identified by the analysis of the glycoform profiles of the secondary PMCA products were western blotted and probed with SHa31 (top panel) and P4 (bottom panel) antibodies. The initial substrate and PrP^{Sc} populations of the primary sPMCAs are indicated at the top of the blots, followed by the substrate used in the secondary sPMCA and the populations identified by the glycoform profiles (figure 5). The PMCA products were digest with 50 µg/ml PK before analysis. The 40, 30 and 20 kDa markers are shown to the left of the blots

Table 7.3. Densitometry of secondary PMCA

Sample[a]	Genotype[b]	SHa31[c]	Positive[d]	P4[e]	Ratio[f]	Normalise[g]	Verdict[h]
$Sc_{2:1}$	V^1V^2	36574.1	Yes	58808.83	0.62	0.2	Scrapie
		31287.27	Yes	60903.61	0.51	0.16	Scrapie
$Int_{2.1}$	V^1V^2	30635.89	Yes	2277.21	13.45	22.98	BSE
		9920.13	No	N/A	N/A	N/A	N/A
$Int_{2:2}$	V^1A^2	29334.04	Yes	2453.03	11.96	3.81	BSE
		21602.43	Yes	953.99	22.64	7.22	BSE
$Sc_{2:2}$	V^1V^2	44420.12	Yes	69430.8	0.64	0.72	Scrapie
		40664.42	Yes	73439.92	0.55	0.62	Scrapie
$Int_{2:3}$	V^1A^2	31635.77	Yes	4087.42	7.74	8.65	BSE
		44960.42	Yes	12726.06	3.53	3.95	BSE
$Sc_{2.3}$	V^1V^2	46445.71	Yes	72961.38	0.64	0.71	Scrapie
		49872.88	Yes	70589.51	0.71	0.79	Scrapie
$BSE_{2:1}$	V^1A^2	39595.57	Yes	11863.69	3.34	3.73	BSE
		36154.42	Yes	8233.97	4.39	4.91	BSE
$BSE_{A2.1}$	A^1V^2	21614.84	Yes	532.26	40.61	54.89	BSE
		27202.25	Yes	1640.77	16.58	22.41	BSE
$BSE_{A2.2}$	A^1A^2	29998.69	Yes	1998.96	15.01	20.28	BSE
		41637.71	Yes	3362.71	12.38	16.74	BSE

a) Specific population of SE1929/0749 analysed by SHa31 and P4 discriminatory western blotting
b) Genotype of substrate on primary and secondary amplification. V designates VRQ/VRQ and A AHQ/AHQ, number refers to on primary or secondary sPMCA
c) Signal of sample when probed with SHa31 primary antibody
d) Positivity of sPMCA reaction. To remove subjective interpretation for a sPMCA product to be considered positive it must exceed a previously generated background value for western blotting (10727.78). “Yes” - indicates threshold value met and replicate is positive, “no” - value not met, as such reaction is not positive and subsequent analysis not applied to that sample.
e) Signal of sPMCA products when probed with P4 primary antibody.
f) Ratio of SHa31:P4 signal
g) Normalisation of ratio by expression as an internal scrapie blotting control (not shown)
h) Normalised ratio indicative of scrapie or BSE. Values lower than 2.9 considered scrapie, greater than 2.9 BSE (Gough et al., 2014).

N/A – Reaction not positive (c) therefore analysis not applied.

7.2.7. Amplification of secondary PMCA populations in alternating substrate sPMCA

To confirm the presence of BSE PrP^{Sc} conformers in the different secondary PMCA populations, the products were used to seed PMCA reactions in the alternating substrate sPMCA assay (chapter 5). This sPMCA assay alternates the ovine substrate between AHQ/AHQ and VRQ/VRQ genotype for each round of PMCA, which preferentially amplifies BSE PrP^{Sc} and has demonstrated 100% sensitivity and specificity when BSE brain homogenates were diluted into scrapie brain homogenates with a final BSE brain content of 0.1% (Simmons et al., 2015). The assay can detect 0.01% BSE in 99.99% scrapie in some cases. This assay was not suitable for application to the primary sPMCA products, as the SE1929/0749 BSE PrP^{Sc} still present in the final round sPMCA products would have seeded reaction. After 5 rounds of amplification the PMCA products were digested with PK, and western blotted before being probed with SHa31 and P4 antibodies (figure 7.7). The core antibody, SHa31, recognises epitopes present in both scrapie and BSE, whereas the epitope for the N-terminal P4 antibody is present in scrapie but lost in BSE PrP^{Sc}. The ratio of signal for these two antibodies are compared in each sample is compared to a standard ratio of 2.9 (three deviations from the mean of a defined scrapie sample) (Gough et al., 2014; Simmons et al., 2015). BSE was identified in both the populations derived from the primary PMCA $BSE_{A1:1}$ population whether the secondary PMCA amplification occurred in either ovine AHQ/AHQ ($BSE_{A2:2}$) or VRQ/VRQ ($BSE_{A2:1}$) (table 7.4). BSE PrP^{Sc} was present in the $BSE_{2:1}$ population derived from primary PMCA $BSE_{1:1}$, however when the substrate for secondary amplification of $BSE_{1:1}$ was VRQ/VRQ and the same sPMCA assay was conducted on the $Sc_{2:3}$ population, BSE was not found to be present. Furthermore in $Sc_{2:3}$ and $Sc_{2:2}$ populations BSE was absent (positive SHa31 PMCA products were identified but with a scrapie like SHa31:P4 ratio). In addition to $BSE_{2:1}$, BSE prion was detected in other populations with a primary VRQ/VRQ PMCA which were $Int_{2:2}$ and $Int_{2:3}$. BSE was not detected in either the $Sc_{2:1}$ or $Int_{2:1}$ populations.

In summary, BSE was absent from all of the secondary PMCA populations in which the genotype of the substrate was VRQ/VRQ for both the primary and secondary PMCAs, and in two of these, $Sc_{2:2}$ and $Sc_{2:3}$, a scrapie like PrP^{Sc} PMCA product was identified. However, for $Int_{2:1}$ which had been amplified in VRQ/VRQ substrate in both primary and secondary sPMCA, BSE prion was absent but the overall P4 antibody reactivity indicated a BSE-like phenotype. All samples with a primary or secondary PMCA that used AHQ/AHQ substrate contained BSE in the secondary PMCA products.

A summary of the different primary and secondary PMCA populations, the genotype of the substrate in both primary and secondary PMCA, and the assays that were applied to them are displayed in figure 7.8.

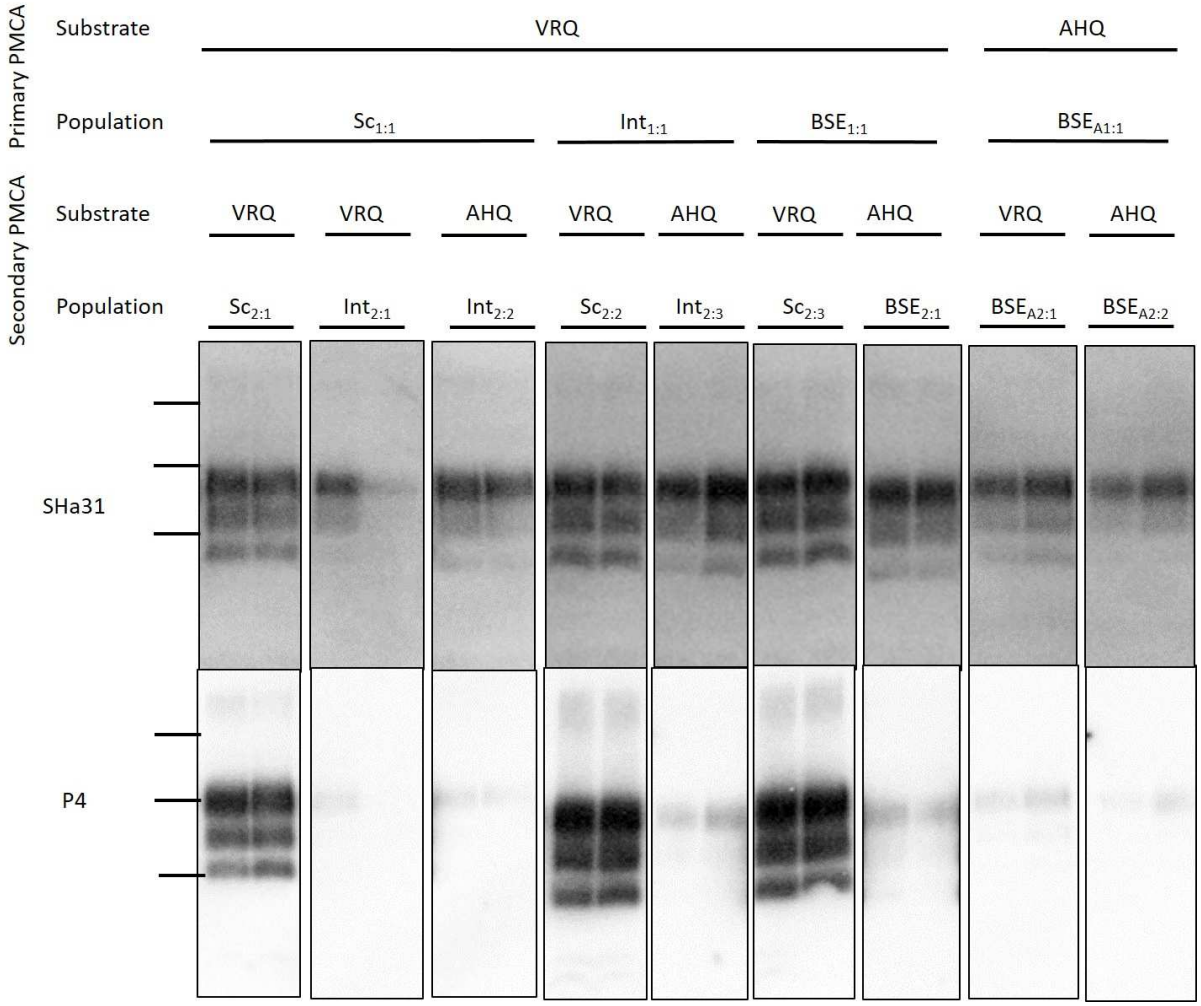


Figure 7.7. Western blots of secondary PMCA products amplified in alternating PMCA assay. Secondary PMCA products were used to seed amplification in the alternating substrate sPMCA assay. In this assay the substrate for amplification was alternated each round between AHQ/AHQ and VRQ/VRQ substrate (*PRNP* at amino acid positions 136, 154 and 171) for a total of 5 rounds. The PMCA products were digested with proteinase K before electrophoresis on SDS-PAGE gels and western blotting. The western blots were then probed with SHa31 and P4 primary antibodies (as indicated). The primary and secondary PMCA populations as well as the substrates in which there were generated are indicated above the blots. The 40, 30 and 20 kDa markers are shown to the left of the blots.

Table 7.4. Densitometry of alternating substrate

Sample[a]	Genotype[b]	SHa31[c]	Positive[d]	P4[e]	Ratio[f]	Normalise[g]	Verdict[h]
$Sc_{2:1}$	V^1V^2	10242.88	No	N/A	N/A	N/A	N/A
		9430.2	No	N/A	N/A	N/A	N/A
$Int_{2.1}$	V^1V^2	10143.13	No	N/A	N/A	N/A	N/A
		9102.08	No	N/A	N/A	N/A	N/A
$Int_{2:2}$	V^1A^2	32424.86	Yes	1938.5	16.73	28.32	BSE
		26254.87	Yes	1916.26	13.7	23.2	BSE
$Sc_{2:2}$	V^1V^2	14416.96	Yes	10251.59	1.41	1.31	Not BSE
		22425.31	Yes	12239.54	1.83	1.71	Not BSE
$Int_{2:3}$	V^1A^2	48027.19	Yes	5047.32	9.52	8.86	BSE
		42687	Yes	4471.83	9.55	8.88	BSE
$Sc_{2.3}$	V^1V^2	21108.8	Yes	10603.77	1.99	1.67	Not BSE
		9739.4	No	N/A	N/A	N/A	N/A
$BSE_{2:1}$	V^1A^2	37007.27	Yes	3991.54	9.27	7.77	BSE
		35640.69	Yes	3285.69	10.85	9.09	BSE
$BSE_{A2.2}$	A^1V^2	41097.2	Yes	1532.89	26.81	37.42	BSE
		42146.52	Yes	2850.76	14.78	20.63	BSE
$BSE_{A2.1}$	A^1A^2	43328.27	Yes	4018.71	10.78	15.05	BSE
		42670.79	Yes	1800.03	23.71	33.08	BSE

a) Specific population of SE1929/0749 analysed by SHa31 and P4 discriminatory western blotting
b) Genotype of substrate on primary and secondary amplification. V designates VRQ/VRQ and A AHQ/AHQ, number refers to on primary or secondary sPMCA
c) Signal of sample when probed with SHa31 primary antibody
d) Positivity of sPMCA reaction. To remove subjective interpretation for a sPMCA product to be considered positive it must exceed a previously generated background value for western blotting (10727.78). “Yes” - indicates threshold value met and replicate is positive, “no” - value not met, as such reaction is not positive and subsequent analysis not applied to that sample.
e) Signal of sPMCA products when probed with P4 primary antibody.
f) Ratio of SHa31:P4 signal
g) Normalisation of ratio by expression as an internal scrapie blotting control (not shown)
h) Normalised ratio indicative of BSE. Values lower than 2.9 considered Not BSE, greater than 2.9 BSE present (Gough et al., 2014; Simmons et al., 2015).

N/A – Reaction not positive (c) therefore analysis not applied.

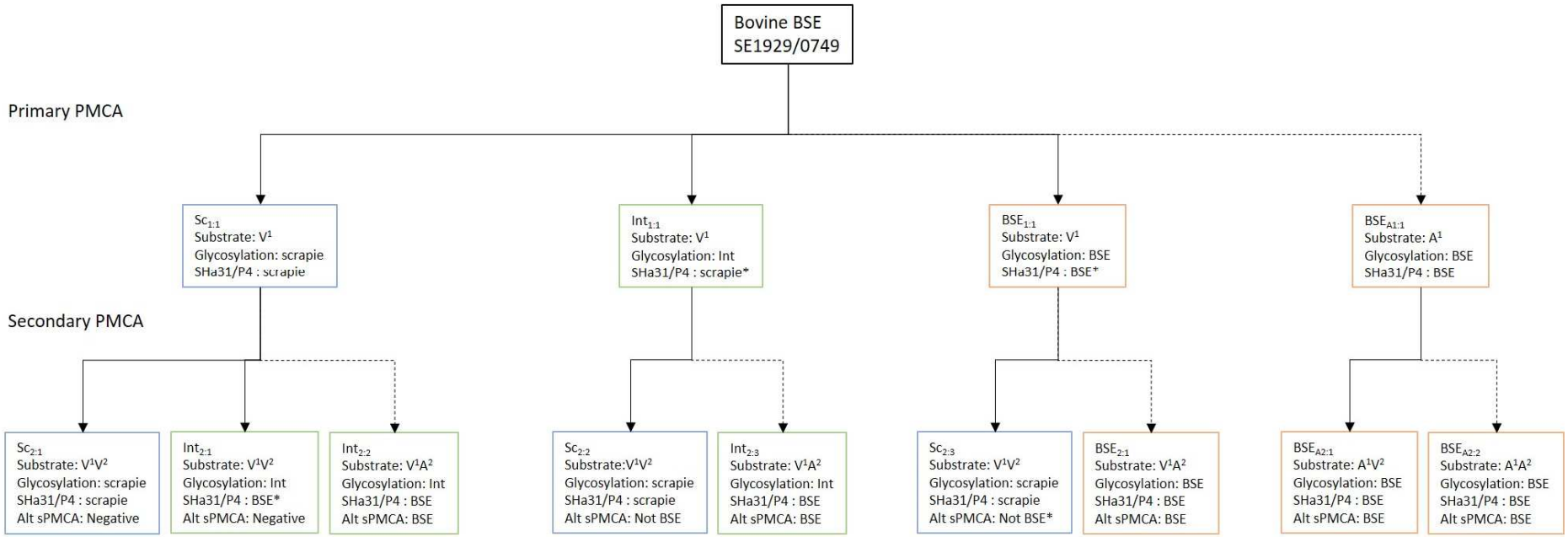


Figure 7.8. Summary of experimental tests applied to primary and secondary PMCA products derived from bovine BSE sample SE1929/0749. Bovine BSE SE1929/0749 was amplified in either VRQ/VRQ or AHQ/AHQ substrate and were organised into populations based on the genotype used for amplification and the glycosylation profile of the products. The PMCA products from each of the primary PMCA populations were used to seed a secondary PMCA, in both VRQ/VRQ and AHQ/AHQ substrates (represented by V or A respectively). The represent a PMCA amplification in VRQ/VRQ (solid line) or AHQ/AHQ (dashed line). Subsequent assays used to characterise these products can be found within each population box. The glycosylation profile of each population is described as either scrapie like (Sc), intermediate (Int) or BSE (BSE). The SHa31/P4 value represents whether the populations reactivity with core (SHa31) and N-terminal (P4) was characteristic of scrapie or BSE. Secondary PMCA products were amplified in alternating substrate sPMCA assay (Alt sPMCA) which is used to detect BSE PrP^{Sc} in excess scrapie PrP^{Sc}. The output of this assay is typically BSE not present (Negative) or BSE present (BSE), yet in this assay positive sPMCA reactions were detected that produced SHa31:P4 ratios typical of scrapie, these results are indicated by “Not BSE”.
*- Only one of two replicates were SHa31 positive for these assays.

7.2.8. Comparing the conformational stability of PrP^{Sc} from different secondary PMCA sub-populations

Conformers of PrP^{Sc} with different secondary structure exhibit different sensitivity to the chaotrophic agent guanidine hydrochloride (Gdn-HCL). As a result of exposure to and subsequent denaturation of PrP^{Sc}, Gdn-HCl can be used to distinguish between BSE and different scrapie strains including CH1641 (Pirisinu et al., 2011). As demonstrated, a spectrum of different PrP^{Sc} has been generated from a single bovine BSE brain pool after passage into VRQ or AHQ substrate by sPMCA. These PrP^{Sc} populations differ in antibody reactivity to core and N-terminal antibodies with some populations resembling scrapie. Furthermore these PrP^{Sc} sub-populations also displayed a range of different glycoform contents some of which are typical of a scrapie isolate and using an assay currently as sensitive to bioassay at detecting BSE, some of these sub-populations did not contain BSE PrP^{Sc}. Therefore to further characterise these populations, conformational stability assays using Gdn-HCl were conducted on a panel of secondary PMCA populations; $Sc_{2:1}$, $Int_{2:1}$, $Int_{2:2}$, $BSE_{A2:1}$ and $BSE_{A2:2}$ (figure 7.11) and also a range of scrapie (figure 7.9) and BSE (figure 7.10) isolate controls. This panel was selected for conformational stability assays, as they are representative of the diversity of different PrP^{Sc} which can be generated by from SE1929/0749. $BSE_{A2:1}$ and $BSE_{A2:2}$ represent typical BSE after initial amplification in AHQ/AHQ substrate. $Sc_{2:1}$, $Int_{2:1}$ and $Int_{2:2}$ are all generated from the same population of primary sPMCA sample, but $Sc_{2:1}$ contains scrapie-like PrP^{Sc} that binds P4 and no BSE PrP^{Sc} can be identified. In contrast PrP^{Sc} present in $Int_{2:1}$ and $Int_{2:2}$ have PrP^{Sc} that is not reactive with P4 antibody, but only $Int_{2:2}$ contains amplifiable BSE PrP^{Sc} in the alternating substrate assay. The signal on western blots probed with SHa31 primary antibody at various concentrations was expressed as a percentage of the baseline signal at 0.5 M Gdn-HCl and plotted in GraphPad Prism.

Two scrapie 10% (w/v) brain homogenates (SE5026/0022 and PG1500/02), sPMCA amplified scrapie (SE5026/0022), two ovine BSE 10% (w/v) brain homogenates (SE1762/0013 and PG0325/10) and sPMCA amplified ovine BSE (SE1762/0013) produced denaturation curves with much variation (figure 7.12A). There was no correlation or clustering of scrapie or BSE isolates as expected from findings in the literature, previously the scrapie has been demonstrated to be much more susceptible to denaturation by Gdn-HCl than BSE (Pirisinu et al., 2011; Vrentas et al., 2013). However, the ovine BSE isolate (PG0325/10) was far more resistant to denaturation which did concur with findings in the literature (Pirisinu et al., 2011).

For the populations of SE1929/0749 produced after sPMCA secondary passage, statistical analysis of the guanidine hydrochloride concentration that produced a 50% reduction in SHa31 signal (Gdn_{50}) (summary of Gdn_{50} values in table 7.7) was conducted using a one-way ANOVA in the statistical

software suite GraphPad Prism which identified a difference in variance (P= 0.0466). Tukey's multiple comparisons of the Gdn_{50} values implied that although there was an overall difference in variance, individual variance between populations could not be deemed significant (Figure 7.13A, table 7.5). However the denaturation from incubation with Gdn-HCl was also interpreted as the percentage decrease in signal at 3.0 M Gdn-HCl ($Gdn_{[3.0]}$), as has been conducted previously (Pirisinu et al., 2011). At this concentration of Gdn-HCl the populations that had been characterised and showed similarities to scrapie in terms of antibody reactivity ($Sc_{2:2}$ and $Int_{2:1}$) were more sensitive to Gdn-HCl, and those with P4 binding indicative of BSE ($Int_{2:1}$, $BSE_{A2:1}$ $BSE_{A2:2}$) in the same tests were less sensitive. The variance in these $Gdn_{[3.0]}$ values was found to be significant in a one-way ANOVA (P= 0.0001) and Tukey multiple comparisons identified populations with scrapie characteristics and those with BSE characteristics as significantly different from each other (figure 13B table 7.6).

The PrP^{Sc} of ovine BSE and scrapie SE1762/0013 and SE5026/0022 were compared before and after amplification in sPMCA using $Gdn_{[3.0]}$ and Gdn_{50} to assess if sPMCA changed the conformational stability of sPMCA using a two tailed unpaired t-test in GraphPad Prism software. For both $Gdn_{[3.0]}$ and Gdn_{50} no significant differences were found for scrapie SE5026/0022 (P =0.568 and P=0.738 respectively), however a significant difference was found between sPMCA amplified PrP^{Sc} and that from brain homogenates (P=0.0013 and P=0.0138) indicating that the conformational stability of bovine BSE significantly changes after amplification in ovine VRQ/VRQ substrate.

SE1929/0749 secondary sPMCA population $Int_{2:1}$ shares common characteristics with scrapie isolate CH1641 such as poor reactivity with primary antibody P4 (Stack et al., 2002). Previously CH1641 has been discriminated from BSE using CSA, to test if this could also be applied to population $Int_{2:1}$ the denaturation of both samples at $Gdn_{[3.0]}$ was compared (figure 7.12C) (Pirisinu et al., 2011). The conformational stability at this concentration of CH1641 and $Int_{2:1}$ was significantly different in an unpaired two-tailed t-test (P=0.0439).

In summary, when interpreting $Gdn_{[3.0]}$ values, the populations of SE1929/0749 generated in two sPMCA reactions did cluster into groups (figure 7.12B), those with relatively high-molecular weights/high P4 binding and associated with scrapie and scrapie-like or intermediate glycoform profiles ($Sc_{2:2}$ and $Int_{2:1}$) were more sensitive to denaturation compared to populations with classical BSE characteristics ($BSE_{A2:1}$ and $BSE_{A2:2}$). $Int_{2:2,}$ gave an intermediate glycoform profile and had BSE-like molecular weight/P4 binding properties but did not have detectable BSE PrP^{Sc} present by the alternating PMCA method; with regard to conformational stability, this sample clustered with the samples that gave typical BSE characteristics.

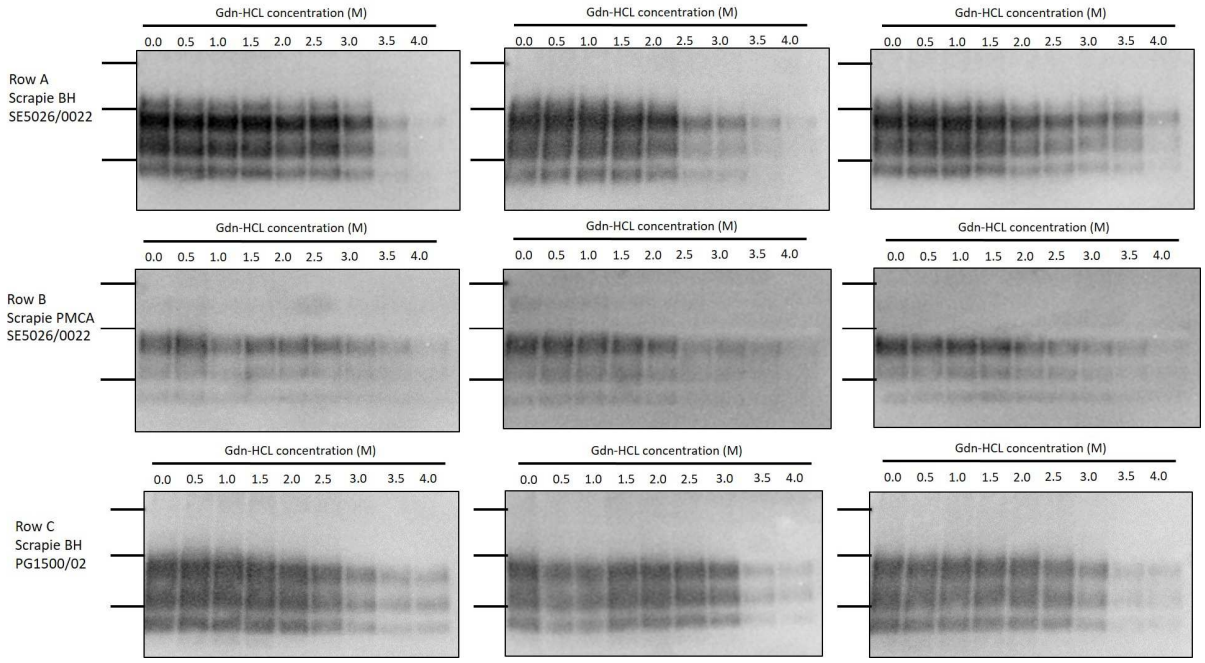


Figure 7.9. Conformational stability assays western blots of scrapie isolates. Two 10% (w/v) brain homogenates of VRQ/VRQ scrapie SE5026/0022 (row **A**) and PG1500/02 (row **C**) as well PG1500/02 after 5 rounds of PMCA in VRQ/VRQ substrate (row **B**) were subjected to denaturation with guanidine hydrochloride (Gdn-HCl). Samples were treated with various concentrations of Gdn-HCl prior to being digested with PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SHa31 antibody. 40, 30 and 20 kDa markers are shown to the left of each blot.

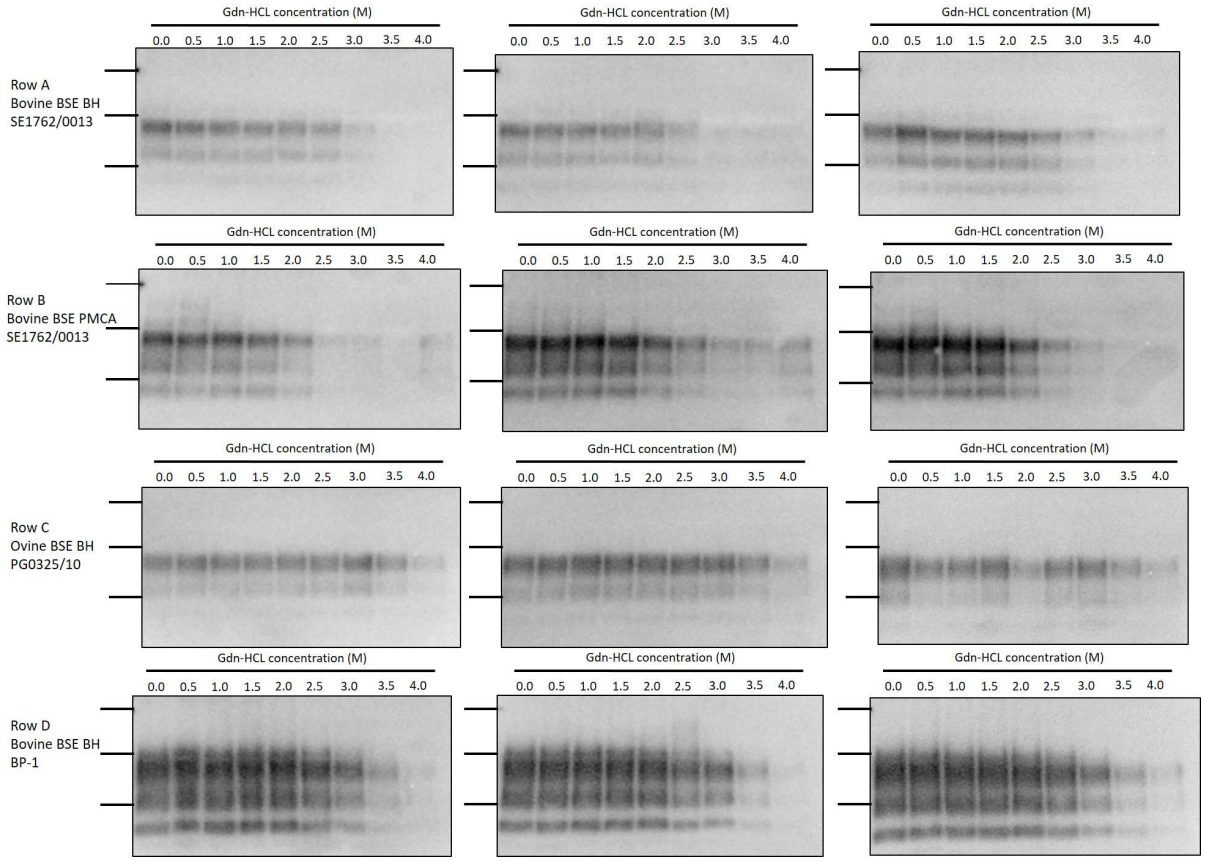


Figure 7.10. Conformational stability assay western blots of BSE samples. Two bovine BSE 10% (w/v) brain homogenates SE1762/0013 (row **A**) and BP-1 (row **D**), SE1762/0013 PMCA products after 5 rounds of amplification in VRQ/VRQ substrate (row **B**) and ovine BSE brain PG0325/10 (row **C**) were subjected to denaturation with guanidine hydrochloride (Gdn-HCl). Samples were treated with various concentrations of Gdn-HCl prior to being digested with PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SHa31 antibody. 40, 30 and 20 kDa markers are shown to the left of each blot.

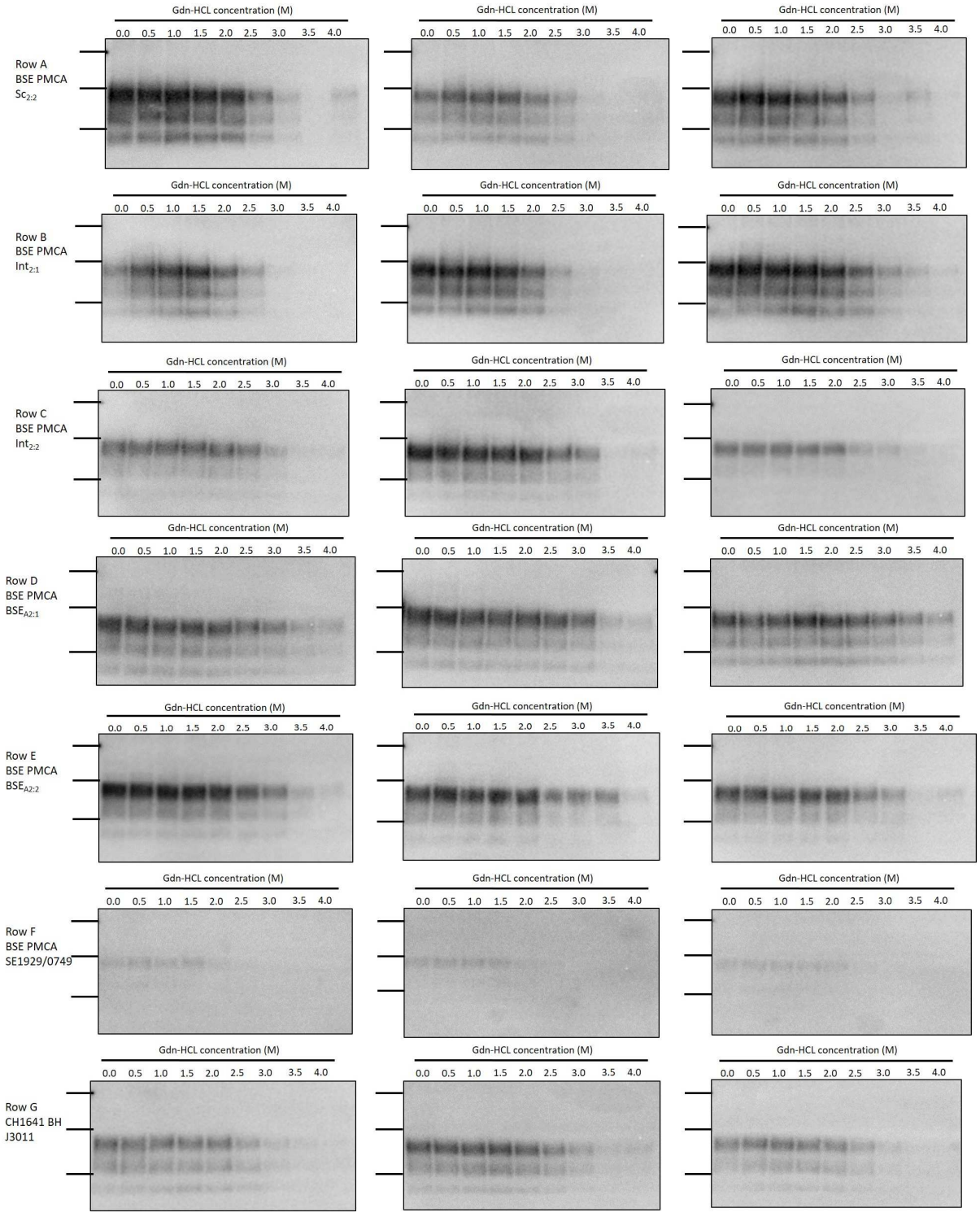


Figure 7.11. Conformational stability assay western blots of secondary PMCA populations and CH1641 scrapie. Secondary PMCA products $Sc_{2:1}$ (row **A**), $Int_{2:1}$ (row **B**), $Int_{2:2}$ (row **C**), $BSE_{A2:1}$ (row **D**), $BSE_{A2:2}$ (row **E**), 1% (w/v) bovine BSE brain homogenate SE1929/0749 (row **E**) and 10% (w/v) CH1641 brain homogenate (row **G**) were subjected to incubation with guanidine hydrochloride (Gdn-HCl). Samples were treated with various concentrations of Gdn-HCl prior to being digested with PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SHa31 antibody. 40, 30 and 20 kDa markers are shown to the left of each blot.

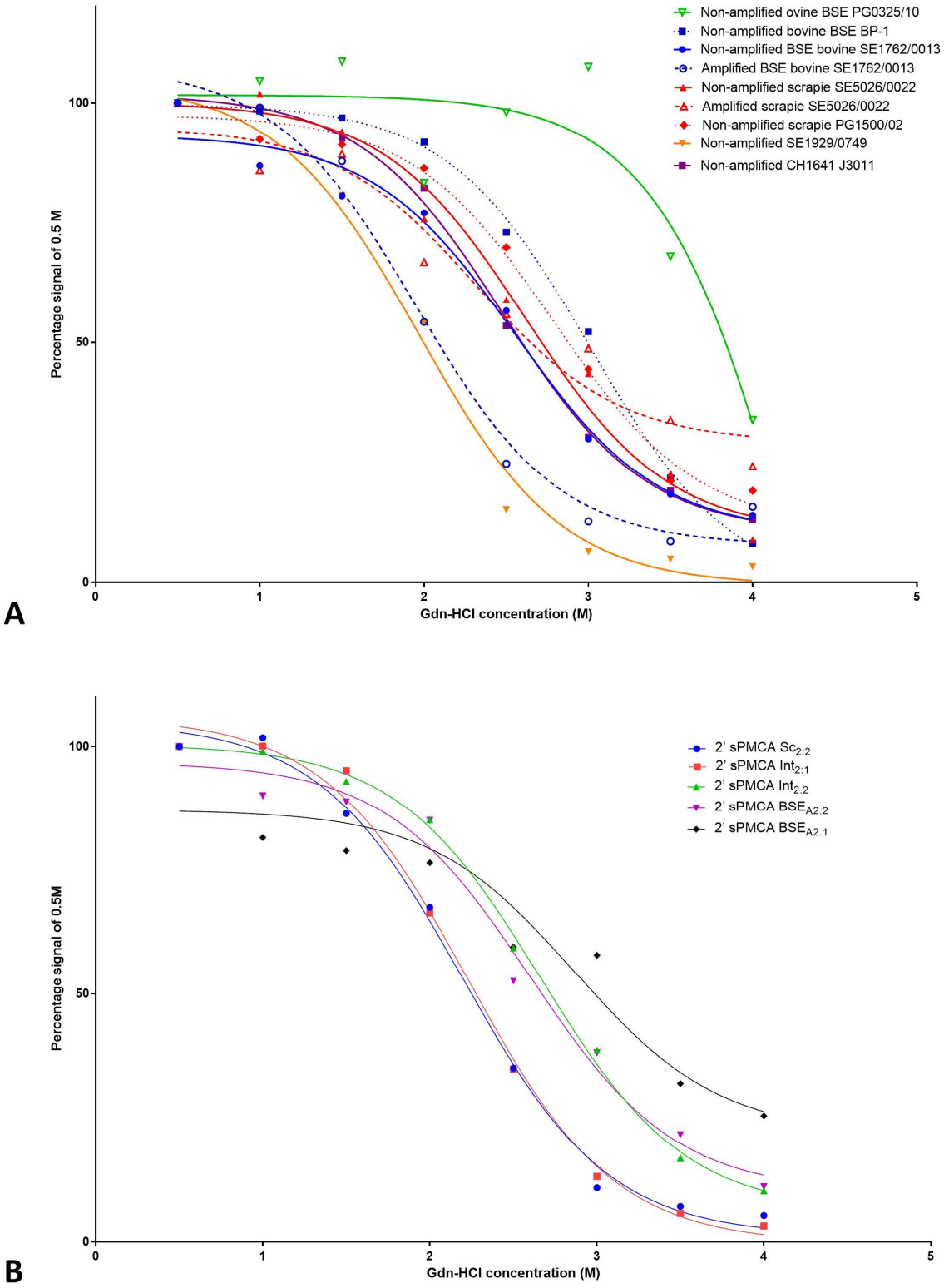


Figure 7.12. Protein degradation of conformational stability assay as a function of signal at 0.5M guanidine hydrochloride (Gdn-HCl). The concentration of Gdn-HCl was plotted against the percentage loss of signal as a measure of denaturation by incubation with Gdn-HCl for scrapie and BSE controls (**A**) and sPMCA generated populations of BSE SE1929/0749 and unamplified

SE1929/0749 and CH1641 10% (w/v) brain homogenates (**B**). Densitometry signals from western blots of samples incubated with Gdn-HCl prior to PK digestion were probed with primary antibody SHa31 were determined (figures 9, 10 and 11). Incubation with 0.5M Gdn-HCl was treated as the negative baseline for denaturation and every subsequent concentration of Gdn-HCl was expressed as a percentage of the 0.5M signal. The lines of best fit were plotted using the sigmoidal dose-response curve function in GraphPad Prism from the three replicates of the denaturation series.

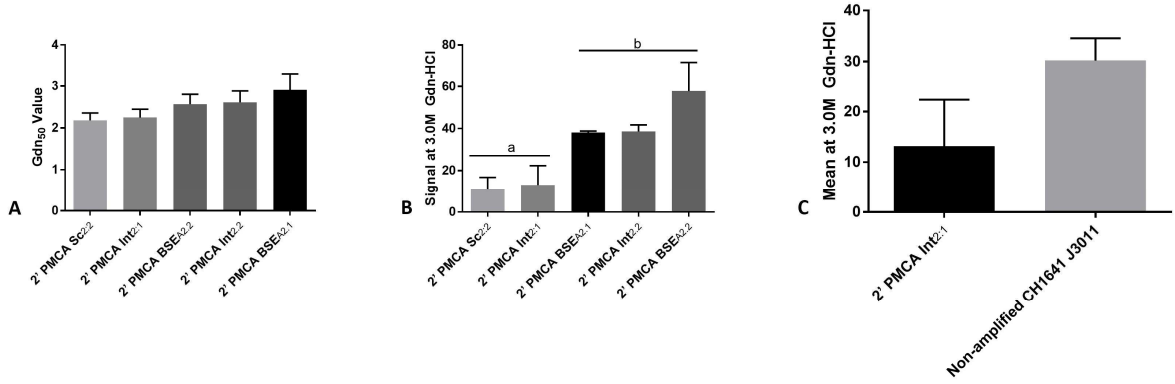


Figure 7.13. Statistical analysis of relative Gdn_{50} and $Gdn_{[3.0]}$ values for secondary PMCA products. Secondary (2') PMCA products ($Sc_{2:2}$, $Int_{2:1}$, $Int_{2:2}$, $BSE_{A2:1}$, $BSE_{A2:2}$) were analysed using various statistical tests in GraphPad Prism. The difference in mean concentration of Gdn-HCl required for a 50% reduction in SHa31 signal on western blots (Gdn_{50}) for the secondary PMCA products was statistically significant using a one-way ANOVA test (P=0.0466)(**A**). The reduction of western blot signal at 3.0M Gdn-HCl ($Gdn_{[3.0]}$) was also tested using a one-way ANOVA and Tukey multiple comparison (**B**), the difference in populations was significant (P=0.0001) while the multiple comparison identified two populations that were significantly different to each other (the bars representing means which share a letter are not significantly different). The $Gdn_{[3.0]}$ of the secondary sPMCA population $Int_{2:1}$ and an isolate of CH1641 (**C**) were compared in a two-tailed unpaired t-Test the difference in means was found to be statistically significant (P=0.439).
All graphs display mean and standard deviation.

Table 7.5. Summary of Tukeys multiple comparisons tests applied the Gdn_{50} of populations of SE1929/0749

Tukey's multiple comparisons test[a]	Mean Diff.[b]	95.00% CI of diff.[c]	Significant[d]	Adjusted P Value[e]
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $Int_{2:1}$	-0.06667	-0.7918 to 0.6585	ns	0.9979
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $Int_{2.2}$	-0.43	-1.155 to 0.2952	ns	0.3526
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $BSE_{A2.1}$	-0.7247	-1.45 to 0.0004925	ns	0.0502
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $BSE_{A2.2}$	-0.3873	-1.112 to 0.3378	ns	0.4451
2' PMCA $Int_{2:1}$ vs. 2' PMCA $Int_{2.2}$	-0.3633	-1.088 to 0.3618	ns	0.5022
2' PMCA $Int_{2:1}$ vs. 2' PMCA $BSE_{A2.1}$	-0.658	-1.383 to 0.06716	ns	0.0801
2' PMCA $Int_{2:1}$ vs. 2' PMCA $BSE_{A2.2}$	-0.3207	-1.046 to 0.4045	ns	0.6098
2' PMCA $Int_{2.2}$ vs. 2' PMCA $BSE_{A2.1}$	-0.2947	-1.02 to 0.4305	ns	0.6765
2' PMCA $Int_{2.2}$ vs. 2' PMCA $BSE_{A2.2}$	0.04267	-0.6825 to 0.7678	ns	0.9996
2' PMCA $BSE_{A2.1}$ vs. 2' PMCA $BSE_{A2.2}$	0.3373	-0.3878 to 1.062	ns	0.5672
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $Int_{2:1}$	-0.06667	-0.7918 to 0.6585	ns	0.9979
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $Int_{2.2}$	-0.43	-1.155 to 0.2952	ns	0.3526
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $BSE_{A2.1}$	-0.7247	-1.45 to 0.0004925	ns	0.0502
2' PMCA $Sc_{2:2}$ vs. 2' PMCA $BSE_{A2.2}$	-0.3873	-1.112 to 0.3378	ns	0.4451
2' PMCA $Int_{2:1}$ vs. 2' PMCA $Int_{2.2}$	-0.3633	-1.088 to 0.3618	ns	0.5022

a) The populations for which the Gdn_{50} is being compared in the multiple comparison
b) The mean difference between Gdn_{50}
c) The difference in the 95% confidence interval of those populations being compared
d) Are the difference in confidence intervals (c) deemed significant (ns –not significant, * - significant)
e) Multiplicity adjusted P value

Table 7.6. Summary of Tukeys multiple comparisons tests applied the percentage denaturation at 3.0 M Gdn-HCl of populations of SE1929/0749

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Adjusted P Value
2' PMCA $Int_{2:1}$ vs. 2' PMCA $Sc_{2:2}$	2.286	-19.32 to 23.89	ns	0.9963
2' PMCA $Int_{2.2}$ vs. 2' PMCA $Sc_{2:2}$	27.79	6.183 to 49.39	*	0.0117
2' PMCA $BSE_{A2.1}$ vs. 2' PMCA $Sc_{2:2}$	27.23	5.626 to 48.83	*	0.0133
2' PMCA $BSE_{A2.2}$ vs. 2' PMCA $Sc_{2:2}$	47.01	25.4 to 68.61	***	0.0002
2' PMCA $Int_{2.2}$ vs. 2' PMCA $Int_{2:1}$	25.5	3.898 to 47.11	*	0.0199
2' PMCA $BSE_{A2.1}$ vs. 2' PMCA $Int_{2:1}$	24.94	3.341 to 46.55	*	0.0227
2' PMCA $BSE_{A2.2}$ vs. 2' PMCA $Int_{2:1}$	44.72	23.12 to 66.33	***	0.0003
2' PMCA $BSE_{A2.1}$ vs. 2' PMCA $Int_{2.2}$	-0.5569	-22.16 to 21.05	ns	>0.9999
2' PMCA $BSE_{A2.2}$ vs. 2' PMCA $Int_{2.2}$	19.22	-2.384 to 40.82	ns	0.0876
2' PMCA $BSE_{A2.2}$ vs. 2' PMCA $BSE_{A2.1}$	19.78	-1.827 to 41.38	ns	0.0769
2' PMCA $Int_{2:1}$ vs. 2' PMCA $Sc_{2:2}$	2.286	-19.32 to 23.89	ns	0.9963
2' PMCA $Int_{2.2}$ vs. 2' PMCA $Sc_{2:2}$	27.79	6.183 to 49.39	*	0.0117
2' PMCA $BSE_{A2.1}$ vs. 2' PMCA $Sc_{2:2}$	27.23	5.626 to 48.83	*	0.0133
2' PMCA $BSE_{A2.2}$ vs. 2' PMCA $Sc_{2:2}$	47.01	25.4 to 68.61	***	0.0002
2' PMCA $Int_{2.2}$ vs. 2' PMCA $Int_{2:1}$	25.5	3.898 to 47.11	*	0.0199

a) The populations for which the Gdn_{50} is being compared in the multiple comparison
b) The mean difference between Gdn_{50}
c) The difference in the 95% confidence interval of those populations being compared
d) Are the difference in confidence intervals (c) deemed significant (ns –not significant, * - significant)
e) Multiplicity adjusted P value

Table 7.7. Summary of Gdn_{50} values generated samples denatured using Gdn-HCl

Sample	Gdn_{50}	Standard error
Non-amplified scrapie SE5026/0022	2.566	0.450
Amplified scrapie SE5026/0022	2.400	0.478
Non-amplified scrapie PG1500/02	2.790	0.173
Non-amplified CH1641 J3011	2.483	0.064
Non-amplified BSE SE1762/0013	2.553	0.181
Amplified BSE SE1762/0013	1.953	0.091
Non-amplified ovine BSE PG0325/10	3.715	0.420
Non-amplified bovine BSE JO pool	2.941	0.095
Non-amplified BSE SE1929/0749	1.917	0.197
2' Passage $Sc_{2:2}$	2.182	0.144
2' Passage $Int_{2:1}$	2.249	0.162
2' Passage $Int_{2:2}$	2.612	0.223
2' Passage $BSE_{2:2}$	2.569	0.192
2' Passage $BSE_{2:1}$	2.907	0.331

7.2.9. Comparison of intermediate population ($Int_{2:2}$) to CH1641 scrapie

The population $Int_{2:1}$ generated from bovine BSE pool SE1929/0749 bears similarity to the scrapie isolate CH1641. Previously $Int_{2:1}$ analysed via western blotting has demonstrated BSE like molecular weight (figure 7.6) and strong core and weak N-terminal antibody reactivity (figure 7.6), yet the alternating sPMCA method able to detect very low levels of ovine BSE (even in the presence of a large excess of ovine scrapie (Gough et al., 2014; Simmons et al., 2015)) indicated an absence of BSE PrP^{Sc}. These characteristics are shared by scrapie isolate CH1641 (Stack et al., 2002). Biochemical techniques have previously been described to differentiate between CH1641 and BSE such as the relative proportions of glycosylated PrP^{Sc} isoforms, however analysis of the glycoform ratio of $Int_{2:1}$ has revealed an intermediate glycoform ratio between BSE and scrapie very similar to those reported for CH1641 (figure 7.5)(Stack et al., 2002). Furthermore the conformational stability of PrP^{Sc} has been used to discriminate between CH1641 and BSE which was confirmed conformational stability assay described here (figure 7.12B) as CH1641 was differentiated from $Int_{2:1}$, using analysis of the $Gdn_{[3.0]}$ by a two-tailed t-test (p=0.0439) (Pirisinu et al., 2011). Probing of CH1641 with the antibody SAF84 has revealed a fragment of PrP^{Sc} with low molecular weight specific to CH1641 (CTF14) (Nicot and Baron,

2010). In an effort to apply a CH1641-specific method to $Int_{2:1}$, secondary sPMCA populations of SE1929/0749 and 4 isolates of CH1641 were probed with SAF84 (figure 7.14). The CTF14 was not detected in any of the CH1641 isolates or secondary PMCA populations.

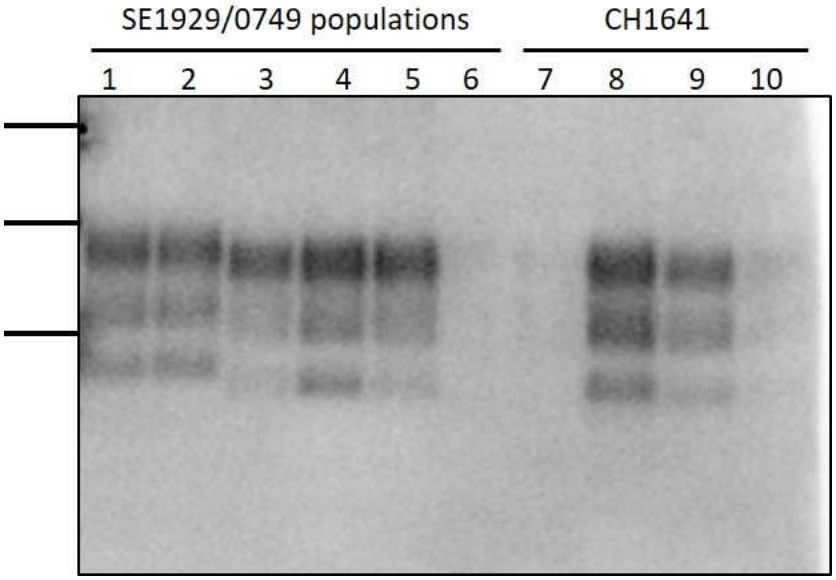


Figure 7.14. Reactivity of SE1929/0749 secondary PMCA populations and CH1641 isolates with primary antibody SAF84. Secondary populations $Sc_{2:2}$, $Int_{2:1}$, $Int_{2:2}$, $BSE_{A2:1}$, $BSE_{A2:2}$ and unamplified SE1929/0749 (lanes 1, 2, 3, 4, 5 and 6 respectively) and CH1641 isolates J2916, J2935, J3011 and KB (lanes 7, 8, 9 and 10) were digested with 50 µg/ml PK, electrophoresed on SDS-PAGE gels, western blotted and probed with SAF84 primary antibody (1 in 1000). 167 µg (CH1641) or 16.7 µg (SE1929/0749) of digested brain material was loaded onto gels. 40, 30 and 20 kDa molecular markers indicated to the left of the western blot.

7.2.10. Amplification of bovine BSE using sPMCA method 2.

The phenomenon of sPMCA amplified BSE sharing molecular characteristics with scrapie has been recently reported in the literature, though there were differences in the sPMCA method used to generate the non-typical BSE PrP^{Sc}, the shift in molecular phenotype only occurred in ovine VRQ/VRQ substrates (designated sPMCA method 2)(Krejciova et al., 2014a). Very subtle changes in sPMCA parameters, such as power output of the sonication horn, temperature, volume of water in the incubator and horn variation over time can produce sPMCAs with different efficiencies. The sPMCA method of Krejciova and co-workers is considerably different to the PMCA method reported here (table 7.8, full details of the PMCA methods can be found in chapter 2.5 and 2.6) and these differences could potentially change the sPMCA efficiency including the switch in molecular phenotype between BSE-like and scrapie-like. The method used in the current study only converted a single bovine BSE sample to a scrapie-like PrP^{Sc}, whereas the method used by Krejciova and co-workers converted all

samples analysed: a single bovine BSE field isolate and isolates of experimental ovine BSE from animals with genotypes of VRQ/VRQ, ARQ/ARQ and ARR/ARR for *PRNP*. To ascertain if using the sPMCA method 2 could more efficiently induce the conversion of bovine BSE PrP^{Sc} to PrP^{Sc} with biochemical characteristics more similar to scrapie, a panel of bovine BSE samples (SE1929/0749, BP-1, SE0253/0055 and SE1762/0013) were subjected to amplification in VRQ/VRQ and AHQ/AHQ substrate (in triplicate) by this method .

Table 7.8. Summary of differences between PMCA methods 1 and 2

Parameter	PMCA Method	
	1	2
Incubation period[a]	29:20	29:40
Sonication time[b]	40	20
Cycles/round[c]	48	96
Rounds[d]	5	9
Dilution[e]	1 in 3	1 in 10
Total sPMCA length[f]	5 days	18 days

a) The incubation time between sonications (minutes:seconds)
b) Duration of sonication period (seconds)
c) Number of cycles of incubation period/sonication period in one round of PMCA
d) Number of rounds per PMCA
e) Dilution of PMCA products into fresh substrate between PMCA rounds.
f) Time taken to complete all rounds of amplification for sPMCA methods

After 9 rounds of sPMCA in method 2 the products were digested with proteinase K before western blotting and subsequently being probed with core and N-terminal antibodies SHa31 and P4. None of the SHa31 sPMCA products which amplified in ovine AHQ/AHQ substrate for any of the 4 BSE samples also reacted with P4 (figure 7.15). However the bovine brain pool SE1929/0749, which previously demonstrated scrapie like P4 reactivity in sPMCA method 1 also demonstrated the same P4 reactivity in 2 positive reactions (figure 7.15). Furthermore the bovine brain homogenate BP-1 also produced PMCA products that showed reactivity with both SHa31 and P4 (figure 7.15). The western blots (figure 7.15) were used to generate a ratio of SHa31:P4 signal in image processing suite ImageJ, which was expressed as a function of an internal blotting control and compared to a previously generated scrapie cut off level of 2.9, samples with a ratio below this were designated as scrapie and those with a ratio above this value were BSE (table 7.9) (Gough et al., 2014; Simmons et al., 2015). The densitometry confirmed that the SE1929/0749 and BP-1 isolates SHa31 and P4 signals were indicative of a scrapie isolate.

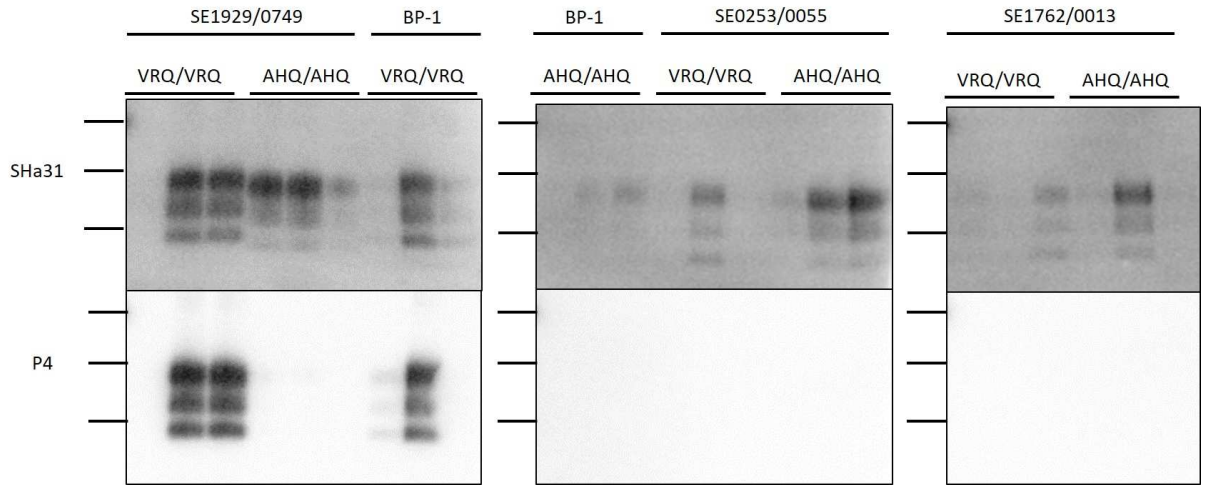


Figure 7.15. Amplification of bovine BSE using PMCA method 2 and reactivity with core and N-terminal antibodies. Four isolates of bovine BSE (SE1929/0749, BP-1, SE0253/0055, and SE1762/0013) were amplified in PMCA method 2 (for summary see table 7.6) for 9 rounds in either ovine VRQ/VRQ or AHQ/AHQ substrate as indicated. Following amplification the round 9 PMCA products were digested with PK at 50 µg/ml and electrophoresed on SDS-PAGE gels before transferral to PVDF membrane. The membranes were then probed with SHa31 (top panel) and P4 (bottom panel) primary antibodies. The 40, 30 and 20 kDa molecular weight markers are indicated to the left of the blots.

Table 7.9. Ratio of signal from PMCA products amplified in method 2 when probed with core and N-terminal antibodies

Sample[a]	Substrate[b]	SHa31[c]	Positive[d]	P4[e]	Ratio[f]	Normalise[g]	Verdict[h]
SE1929/0749	VRQ/VRQ	3472.41	No	N/A	N/A	N/A	N/A
		40346.79	Yes	60344.14	0.67	0.76	Scrapie
		39107.91	Yes	60305.66	0.65	0.74	Scrapie
	AHQ/AHQ	31643.43	Yes	754.41	41.94	47.71	BSE
		32210.58	Yes	431.26	74.69	84.95	BSE
		13775.09	Yes	16.54	833.04	947.51	BSE
BP-1	VRQ/VRQ	6526.01	No	N/A	N/A	N/A	N/A
		36605.03	Yes	46513.17	0.79	0.90	Scrapie
		9589.61	No	N/A	N/A	N/A	N/A
	AHQ/AHQ	4520.03	No	N/A	N/A	N/A	N/A
		5874.17	No	N/A	N/A	N/A	N/A
		6900.43	No	N/A	N/A	N/A	N/A
SE0253/0055	VRQ/VRQ	1414.18	No	N/A	N/A	N/A	N/A
		13021.87	Yes	1.00	13021.87	12204.72	BSE
		1555.00	No	N/A	N/A	N/A	N/A
	AHQ/AHQ	5281.17	No	N/A	N/A	N/A	N/A
		21366.89	Yes	1.00	21366.89	20026.07	BSE
		29333.91	Yes	1.00	29333.91	27493.15	BSE
SE1762/0013	VRQ/VRQ	4916.32	No	N/A	N/A	N/A	N/A
		2668.27	No	N/A	N/A	N/A	N/A
		10311.31	No	N/A	N/A	N/A	N/A
	AHQ/AHQ	3873.98	No	N/A	N/A	N/A	N/A
		22842.69	Yes	1.00	22842.69	21552.54	BSE
		2834.06	No	N/A	N/A	N/A	N/A

a) Bovine BSE sample amplified in PMCA and analysed by SHa31 and P4 discriminatory western blotting
b) Substrate sample was amplified in
c) Signal of sample when probed with SHa31 primary antibody
d) Positivity of sPMCA reaction. To remove subjective interpretation for a sPMCA product to be considered positive it must exceed a previously generated background value for western blotting (10727.78). “Yes” - indicates threshold value met and replicate is positive, “no” - value not met, as such reaction is not positive and subsequent analysis not applied to that sample.
e) Signal of sPMCA products when probed with P4 primary antibody. P4 values of 1.00 represent undetectable levels of PrP^{Sc} when probed with P4 primary antibody
f) Ratio of SHa31:P4 signal
g) Normalisation of ratio by expression as an internal scrapie blotting control (not shown)
h) Normalised ratio indicative of scrapie or BSE. Values lower than 2.9 considered scrapie, greater than 2.9 BSE (Gough et al., 2014; Simmons et al., 2015).

N/A – Reaction not SHa31 positive (c) therefore analysis not applied.

7.2.11. BSE content in round 9 products generated using PMCA method 2

To assess if BSE PrP^{Sc} was still present in the PMCA products after 9 rounds of amplification, the alternating substrate sPMCA assay was applied to the round 9 samples. In this assay the genotype of the substrate used for amplification is switched between ovine AHQ/AHQ and VRQ/VRQ for each round, which preferentially amplifies BSE. To further ensure all positive PMCA products are indeed BSE they are also probed with SHa31 and P4 primary antibodies (figure 7.16) and the ratio of signal for the two antibodies used to assess if the PrP^{Sc} is BSE (table 7.10).

BSE was present in all of the isolates when amplified in AHQ/AHQ except one replicate of BP-1 which was negative for both SHa31 and P4 reactivity (figure 7.16). The three replicates of SE0253/0055 and SE1762/0013 amplified in VRQ/VRQ substrates were positive and demonstrated SHa31:P4 ratio typical of BSE. Two of the three isolates for both SE1929/0749 and BP-1 amplified in VRQ/VRQ also had SHa31:P4 ratios typical of BSE. For BP-1 both of the replicates had SHa31:P4 ratios characteristic of BSE and including the replicate that produced a scrapie like signal, indicating that either any molecular switch occurring in the PrP^{Sc} was transient and could be reversed during the alternate substrate PMCA method, or independent BSE PrP^{Sc} prions still were present in the BP-1 isolate which had a dominant PrP^{Sc} type gave a scrapie like antibody binding ratio. This is unlikely as only 1 pg of original brain material was analysed in western blotting which is significantly lower than the sensitivity of western blotting in our hands (10-30 ng). One of the SE1929/0749 isolates that was positive for SHa31 also produced a strong P4 signal, while one did not, indicating one replicate contained BSE PrP^{Sc} and one replicate did not. Both of these replicates before amplification in alternating substrate assay had scrapie like ratios, indicating that in at least one of these, BSE PrP^{Sc} was still present.

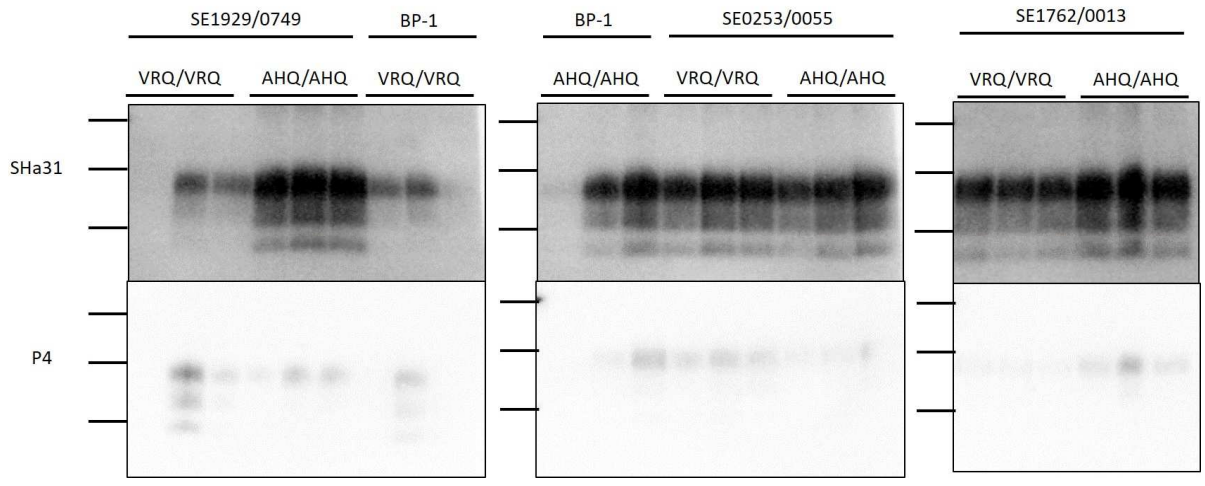


Figure 7.16. Western blots of PMCA method 2 products amplified in alternating substrate assay. 10 µl of PMCA products from each replicate of PMCA method 2 amplified bovine BSE (SE1929/0749, BP-1, SE0253/0055 and SE1762/0013 as indicated) was used to seed a fresh amplification, diluted 1 in 10 in ovine AHQ/AHQ substrate. The PMCA was ran for 5 rounds with a 1

in 3 dilution of seed into fresh substrate and the genotype of the substrate for amplification alternated between ovine AHQ/AHQ and VRQ/VRQ at each round. The PMCA products were digested with 50 µg/ml PK before SDS-PAGE and transferral to PVDF membranes. The membranes were probed with either SHa31 (top panel) or P4 (bottom panel) primary antibodies. The 40, 30 and 20 kDa markers are indicated to the right of the blots.

Table 7.10. Ratio of signal from PMCA products amplified in method 2 followed by amplification in alternating substrate method then probed with core and N-terminal antibodies.

Sample[a]	Substrate[b]	SHa31[c]	Positive[d]	P4[e]	Ratio[f]	Normalise[g]	Verdict[h]
SE1929/0749	VRQ/VRQ	1111.01	No	N/A	N/A	N/A	N/A
		21917.58	Yes	11061.61	1.98	1.63	BSE absent
		19272.43	Yes	2411.28	7.99	6.59	BSE present
	AHQ/AHQ	50187.73	Yes	1130.99	44.38	36.57	BSE present
		59090.17	Yes	2559.28	23.09	19.03	BSE present
		55126.12	Yes	2620.03	21.04	17.34	BSE present
BP-1	VRQ/VRQ	17633.83	Yes	474.26	37.18	30.64	BSE present
		20927.95	Yes	4355.51	4.80	3.96	BSE present
		5735.07	No	N/A	N/A	N/A	N/A
	AHQ/AHQ	5772.36	No	N/A	N/A	N/A	N/A
		35079.43	Yes	1385.82	25.31	28.89	BSE present
		55415.14	Yes	4579.25	12.10	13.81	BSE present
SE0253/0055	VRQ/VRQ	44573.22	Yes	2425.21	18.38	20.97	BSE present
		54164.07	Yes	3194.47	16.96	19.35	BSE present
		50755.54	Yes	2725.38	18.62	21.25	BSE present
	AHQ/AHQ	38765.91	Yes	1081.99	35.83	40.89	BSE present
		46347.59	Yes	1272.58	36.42	41.56	BSE present
		51952.90	Yes	1602.65	32.42	36.99	BSE present
SE1762/0013	VRQ/VRQ	37036.40	Yes	501.68	73.83	77.41	BSE present
		35155.19	Yes	408.68	86.02	90.20	BSE present
		38451.35	Yes	388.68	98.93	103.73	BSE present
	AHQ/AHQ	53201.25	Yes	1658.01	32.09	33.64	BSE present
		54133.78	Yes	3892.95	13.91	14.58	BSE present
		45406.59	Yes	1221.06	37.19	38.99	BSE present

a) Bovine BSE sample amplified in PMCA and analysed by SHa31 and P4 discriminatory western blotting
b) Substrate sample was originally amplified in
c) Signal of sample when probed with SHa31 primary antibody
d) Positivity of sPMCA reaction. To remove subjective interpretation for a sPMCA product to be considered positive it must exceed a previously generated background value for western blotting (10727.78). “Yes” - indicates threshold value met and replicate is positive, “no” - value not met, as such reaction is not positive and subsequent analysis not applied to that sample.
e) Signal of sPMCA products when probed with P4 primary antibody.
f) Ratio of SHa31:P4 signal
g) Normalisation of ratio by expression as an internal scrapie blotting control (not shown)
h) Normalised ratio indicative of BSE. Values lower than 2.9 considered Not BSE, greater than 2.9 BSE present (Gough et al., 2014; Simmons et al., 2015).

N/A – Reaction not SHa31 positive (c) therefore analysis not applied.

7.2.12. Proportion of PrP^{Sc} glycosylated isoforms present in PMCA 2 amplified bovine BSE.

Four reactions were SHa31 positive after being amplified in PMCA 2 in VRQ/VRQ substrate, two SE1929/0749 replicates, one replicate of BP-1 and one replicate of SE0253/0055. The replicate of SE0253/0055 was the only replicate which had a SHa31/P4 binding ratio indicative of BSE, the other two isolates gave strong signals when probed with P4 (table 7.8). The glycosylation profiles of these three isolates correlated with the P4 reactivity. The SE0253/0055 replicate had a typical BSE ratio and clustered with the BSE controls when di-glycosylated content was plotted against the mono-glycoyslated protein. However the replicates of SE1929/0749 and BP-1 correlated with the scrapie control isolates with much more equal levels of both PrP^{Sc} glycosylated isoforms (figure 7.17A). In contrast, the two positive replicates of SE0253/0055 and the single replicate of SE1762/0013 that were amplified in AHQ/AHQ substrate had typical BSE glycoforms, as did two of the three positive SE1929/0749 replicates in this substrate. The other SE1929/0749 replicate produced an intermediate glycoform ratio between both BSE and scrapie control groups (figure 7.17B).

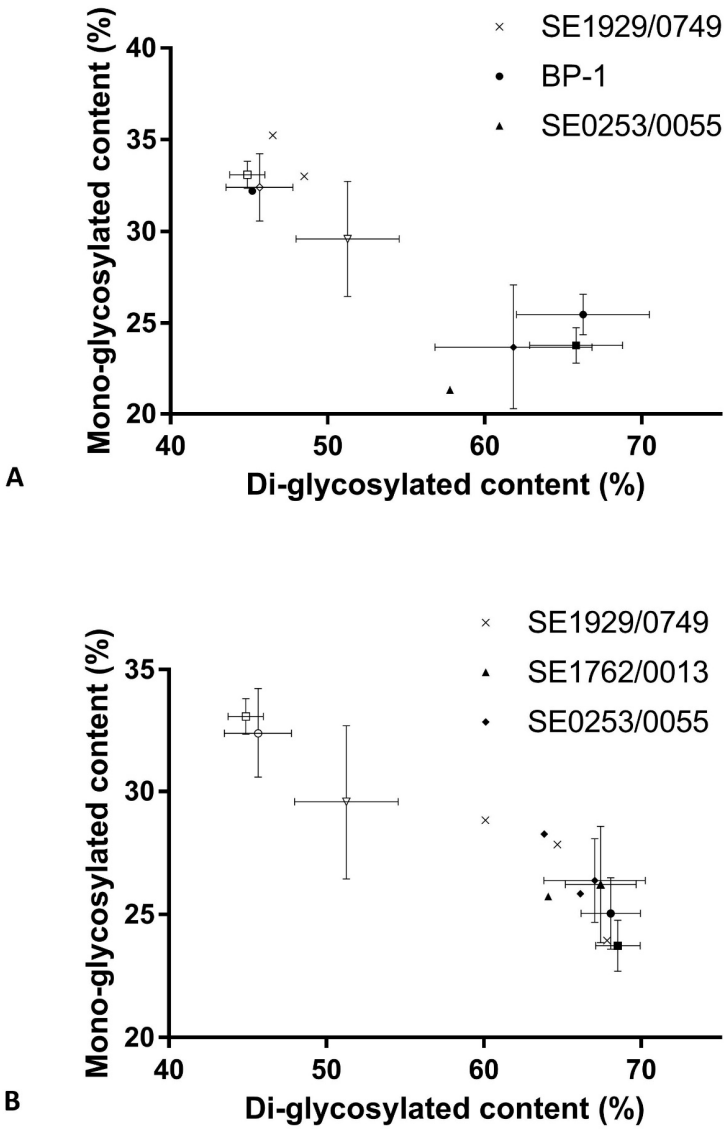


Figure 7.17. Glycoform profiles of bovine BSE isolates amplified in PMCA method 2 in either ovine VRQ/VRQ or AHQ/AHQ substrate. All of the positive SHa31 replicates of the bovine BSE isolates SE1929/0749, BP-1, SE0253/0055 and SE1762/0013 amplified in both ovine VRQ/VRQ (**A**) and AHQ/AHQ (**B**) substrates were analysed in ImageJ processing suite for the relative proportions of the PrP^{Sc} glycosylated isoforms. The relative abundance of the di-glycosylated and mono-glycosylated PrP^{Sc} was expressed as a percentage of the total signal for the 3 isoforms and then plotted against each other. In A, non-filled symbols with standard deviations are scrapie sPMCA amplified controls; square - PG1276/02, triangle - PG1275/02, diamond - SE5026/0022. Filled symbols with standard deviations depict BSE sPMCA amplified controls; square - FT5026/0037, circle - SE1762/0007 and diamond - SE1762/0013. In B, scrapie sPMCA amplified controls are represented by non-filled symbols with standard deviations; square - PG1276/02, triangle - PG1275/02, diamond - SE5026/0022. Filled symbols with standard deviations depict BSE sPMCA amplified controls; square – FT5026/0037, circle SE1762/0007, diamond - SE1762/0013 and triangle - SE5023/0055.

7.2.13. Individual sPMCA round analysis of SE1929/0749 and BP-1

Both bovine BSE isolates SE1929/0749 and BP-1 seed reactions in ovine VRQ/VRQ substrate and form PrP^{Sc} with scrapie-like high molecular weight, low levels of di-glycosylated form and high primary antibody P4 reactivity. To determine when these changes in PrP^{Sc} conformers arise in the amplification process, each round of sPMCA was digested and probed with SHa31 and P4 antibodies (figure 7.18). For SE1929/0749 the aberrant conformers arise between rounds 5 and 6, and appear in round 7 in BP-1 amplification. In SE1929/0749 the classical BSE conformers do not appear to form, whereas in BP-1 they clearly are visible in the seed at round 1 before the signal peters out between rounds 2 and 5. The difference in visibility of the seed between these two isolates likely lies in the difference in concentration of the brain homogenates, SE1929/0749 is a 1% (w/v) homogenate whereas BP-1 is a 20% (w/v) homogenate.

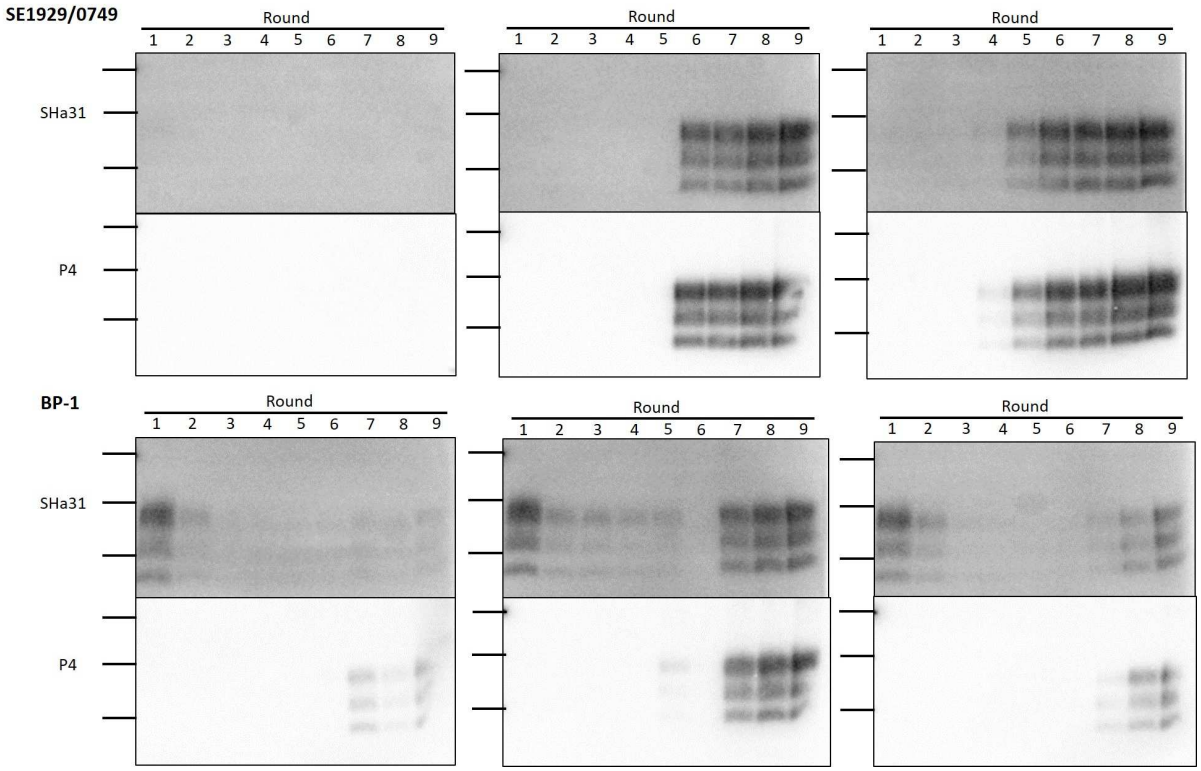


Figure 7.18. Western blotting of rounds 1 to 9 of both SE1929/0749 and BP-1 during amplification in VRQ/VRQ substrate using sPMCA method 2. For each of the triplicate amplifications of SE1929/0749 and BP-1, each PMCA round was digested, separated by SDS-PAGE, transferred onto membranes and probed with both primary antibodies SHa31 (top panel) and P4 (bottom panel). The molecular markers are indicated to the left of each western blot, which have molecular weights of 40, 30 and 20 kDa.

7.2.14. Amplification of individual isolates within BP-1 pooled brain sample using PMCA method 2

BP-1 is a brain pool made up of 10 single bovine brain homogenates (table 7.1) that were available for this study. Under routine sPMCA conditions used in this thesis the BP-1 pool amplified faithfully as a BSE isolate without showing any P4 antibody reactivity or high molecular weight PMCA products. However using PMCA method 2 in VRQ/VRQ substrate, based on work by Krejciova *et al* 2014, BP-1 formed PrP^{Sc} with high molecular weights, glycosylation patterns and P4 reactivity associated with scrapie.

The phenomenon of BSE molecular plasticity occurs during VRQ/VRQ amplification and was observed in 4 isolates by Krejciova and co-workers, 3 ovine BSE isolates and a single BSE isolate. However, given that in the present study only 2 out of 4 bovine BSE samples demonstrated this switch in molecular phenotype, it is important to ascertain the frequency of the molecular plasticity shown by BSE isolates. Therefore the 10 individual bovine BSE isolates comprising BP-1 were amplified independently in VRQ/VRQ substrate using sPMCA method 2 for 9 rounds before digestion, western blotting and discriminatory SHa31 and P4 antibody profiling (figure 7.19, table 7.10).

Three isolates failed to amplify PrP^{Sc} that was detected by SHa31 in any of the assay triplicates (PG00034/04, PG00354/03 and 00365/03). A total of 15 replicates of the remaining 7 bovine BSE isolates were SHa31 positive, of these 14 also reacted with P4 (table 7.11). Two replicates of PG00487/03 were SHa31 positive but both clearly display different molecular weights, and only one of these replicates produces P4 signal (figure 7.19). This would indicate that even within a single bovine BSE isolate the potential exists to form PrP^{Sc} with different conformations and inherent biochemical characteristics.

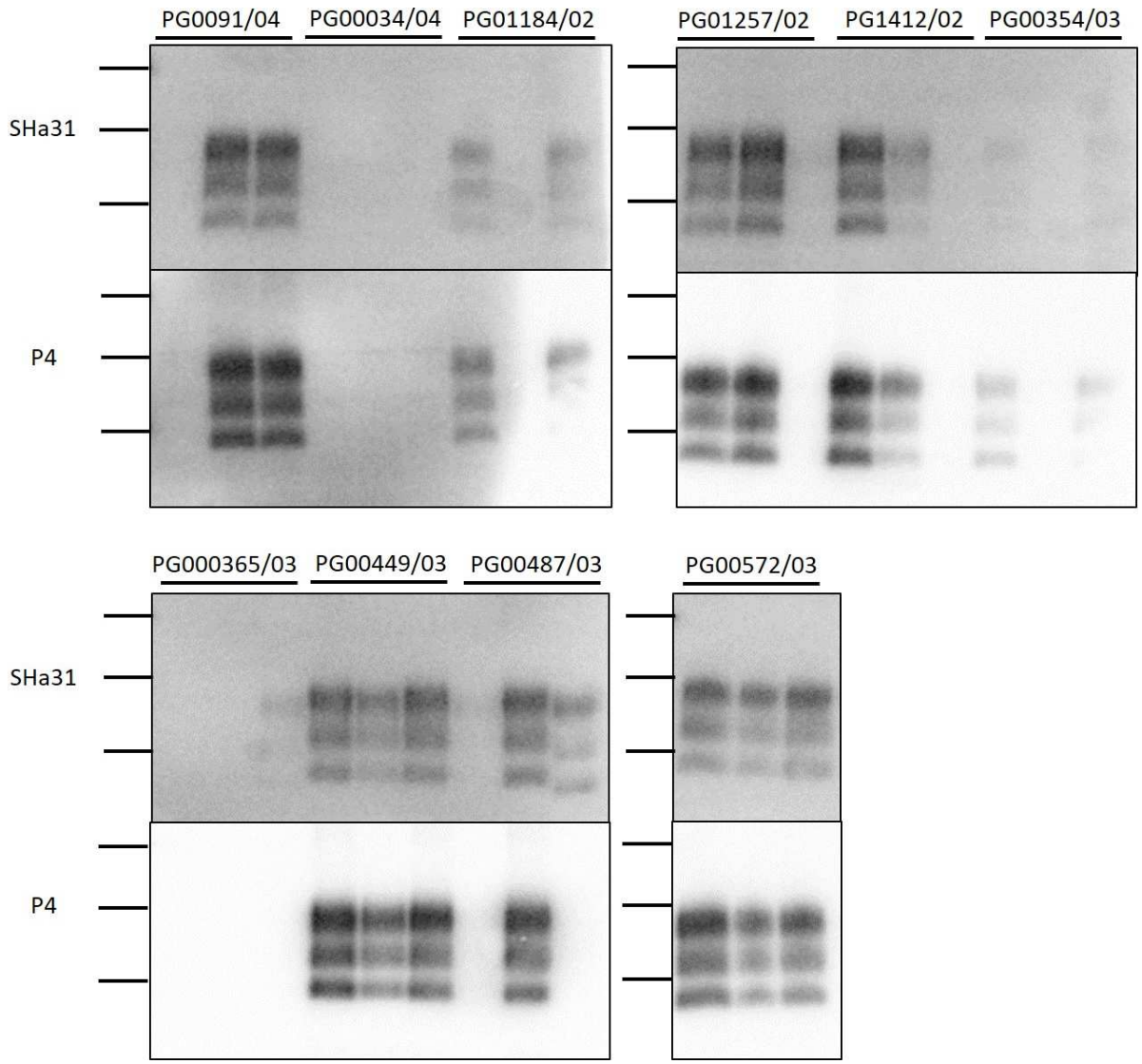


Figure 7.19. Amplification of constituents BP-1 single isolates in VRQ/VRQ substrate using PMCA method 2 followed by probing with core and N-terminal antibodies. The 10 bovine BSE isolates that were pooled into BP-1 were amplified individually in PMCA method 2 for 9 rounds. The isolates are PG0091/04, PG00034/04, PG01184/02, PG01257/02, PG1412/02, PG00354/03, PG000365/03, PG00449/03, PG00487/03 and PG00572/03. Following amplification the PrP^{Sc} was digested with 50 µg/ml PK and separated in SDS-PAGE before transfer to PVDF membrane and probing with primary antibodies SHa31 (top panel) and P4 (bottom panel). The molecular markers are indicated to the left of each western blot, which have molecular weights of 40, 30 and 20 kDa.

Table 7.11. SHa31 and P4 ratios of individual BP-1 PMCA products

Sample[a]	SHa31[b]	Positive[c]	P4[d]	Ratio[e]	Normalise[f]	Verdict[g]
Bovine BSE PG0091/04	2267.82	No	N/A	N/A	N/A	N/A
	35500.86	Yes	44015.56	0.81	0.90	Scrapie
	34846.79	Yes	39750.46	0.88	0.98	Scrapie
Bovine BSE PG00034/04	1740.88	No	N/A	N/A	N/A	N/A
	1939.64	No	N/A	N/A	N/A	N/A
	851.03	No	N/A	N/A	N/A	N/A
Bovine BSE PG01184/02	13393.34	Yes	22404.62	0.60	0.67	Scrapie
	3036.82	No	N/A	N/A	N/A	N/A
	10667.74	No	N/A	N/A	N/A	N/A
Bovine BSE PG01257/02	30941.76	Yes	38862.30	0.80	0.89	Scrapie
	41012.64	Yes	45382.81	0.90	1.01	Scrapie
	6660.87	No	N/A	N/A	N/A	N/A
Bovine BSE PG1412/02	39884.64	Yes	49190.35	0.81	0.91	Scrapie
	14143.53	Yes	19290.56	0.73	0.82	Scrapie
	1815.68	No	N/A	N/A	N/A	N/A
Bovine BSE PG00354/03	4645.19	No	N/A	N/A	N/A	N/A
	855.45	No	N/A	N/A	N/A	N/A
	1048.96	No	N/A	N/A	N/A	N/A
Bovine BSE PG00365/03	1513.00	No	N/A	N/A	N/A	N/A
	1716.18	No	N/A	N/A	N/A	N/A
	4688.11	No	N/A	N/A	N/A	N/A
Bovine BSE PG00449/03	29041.27	Yes	58368.12	0.50	0.56	Scrapie
	20602.99	Yes	40856.10	0.50	0.56	Scrapie
	28894.89	Yes	50433.95	0.57	0.64	Scrapie
Bovine BSE PG00487/03	4164.02	No	N/A	N/A	N/A	N/A
	33088.15	Yes	52444.68	0.63	0.71	Scrapie
	17921.11	Yes	1452.65	12.34	13.81	BSE
Bovine BSE PG00572/03	31037.40	Yes	53636.32	0.58	0.65	Scrapie
	19783.87	Yes	31053.50	0.64	0.71	Scrapie
	26281.23	Yes	37120.86	0.71	0.79	Scrapie

a) Individual bovine BSE isolates from BP-1 analysed by SHa31 and P4 discriminatory western blotting
b) Signal of sample when probed with SHa31 primary antibody
c) Positivity of sPMCA reaction. To remove subjective interpretation for a sPMCA product to be considered positive it must exceed a previously generated background value for western blotting (10727.78). “Yes” - indicates threshold value met and replicate is positive, “no” - value not met, as such reaction is not positive and subsequent analysis not applied to that sample.
d) Signal of sPMCA products when probed with P4 primary antibody.
e) Ratio of SHa31:P4 signal
f) Normalisation of ratio by expression as an internal scrapie blotting control (not shown)
g) Normalised ratio indicative of scrapie or BSE. Values lower than 2.9 considered scrapie, greater than 2.9 BSE (Gough et al., 2014; Simmons et al., 2015).

N/A – Reaction not positive (c) therefore analysis not applied.

7.2.15. Identifying possible ovine contaminants in SE1929/0749

The phenomenon of a bovine BSE isolate undergoing a drastic change in biochemical features, after amplification in VRQ/VRQ, which fit more closely with those of a scrapie isolate is a finding with significant implications for our understanding of prion misfolding with ramifications for human and animal health. However, the apparent formation of a scrapie like conformer of BSE could also be explained by a scrapie contaminant in the bovine BSE pool, which in turn outcompetes the BSE PrP^{Sc} in VRQ/VRQ amplification and becomes the dominant conformer in a sPMCA reaction. While this explanation seems unlikely when the molecular switch has been observed in different bovine BSE isolates on separate occasions, including by independent researchers at a different institution: one bovine brain pool SE1929/0749 that was produced at APHA, 7 of the bovine isolates which were used to create BP-1 (individual isolates and the pool produced) and independently by Krejciova and co-workers (Krejciova et al., 2014b).

None the less, a panel of 3 VRQ/VRQ scrapie isolates were diluted into BP-1 and amplified by sPMCA in ovine VRQ/VRQ substrate for 5 rounds (PMCA method 1). The sPMCA products from a seed that contained 0.01% (v/v) scrapie and 99.99% (v/v) BSE were probed with SHa31 and P4 (figure 7.20). PG1275/02 and PG1276/02 at this dilution did not appear to amplify and the PMCA products do not react with P4 antibody. However, 0.01% (v/v) of scrapie isolate SE5026/0022 in the seed amplified and was recognised by P4, indicating this isolate of scrapie replicated particularly well in this sPMCA, the un-glycosylated band when probed with SHa31 appeared to be a 'hybrid' with both higher molecular weight scrapie-like and lower molecular weight BSE-like bands present. Therefore, none of the three scrapie isolates at this concentration in the spike can generate sPMCA products that resemble the high molecular weight products observed in SE1929/0749.

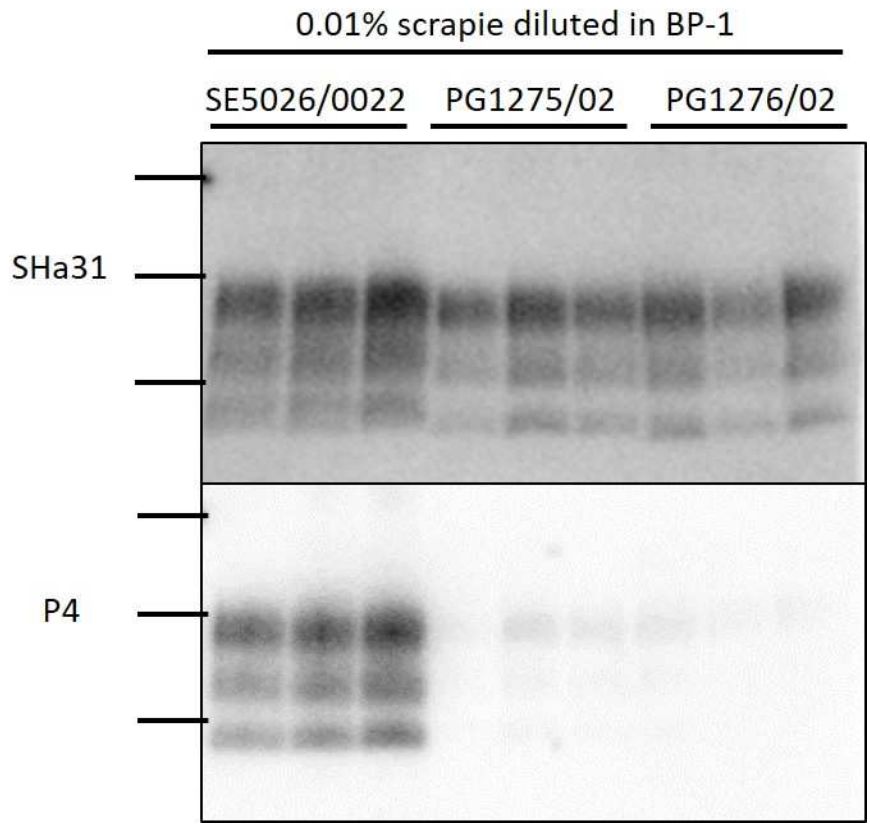


Figure 7.20. Scrapie and BSE mixes amplified in sPMCA and probed with core and N-terminal antibodies. Spikes for PMCA contained 0.01% (v/v) SE5026/0022, PG1275/02 or PG1276/02 and 99.99% (v/v) bovine BSE BP-1 as indicated. Spikes were amplified for 5 rounds in ovine VRQ/VRQ substrate, digested with PK, electrophoresed on SDS-PAGE, transferred to PVDF membranes and treated with SHa31 (top panel) and P4 (bottom panel) primary antibodies. The 40, 30 and 20 kDa markers are displayed to the right of the blots.

Next, bovine and ovine DNA were extracted from brain homogenates along with DNA from SE1929/0749 to assess if ovine DNA content in an assay with sensitivity of detecting at least 0.01%.ovine DNA in bovine DNA. Dilutions series of ovine DNA diluted into bovine DNA which were subjected to PCR along with ovine specific primers for 45 cycles were ran on 2% agarose gels and visualised using a trans illuminator. Although this revealed a good level of sensitivity of the PCR, able to detect 0.0125% ovine DNA (figure 7.22B), cross reactivity of ovine DNA with bovine primers (figure 7.21A 2) meant that non-specific amplification of ovine DNA with bovine specific primers could not be ruled out as an explanation for the PCR products.

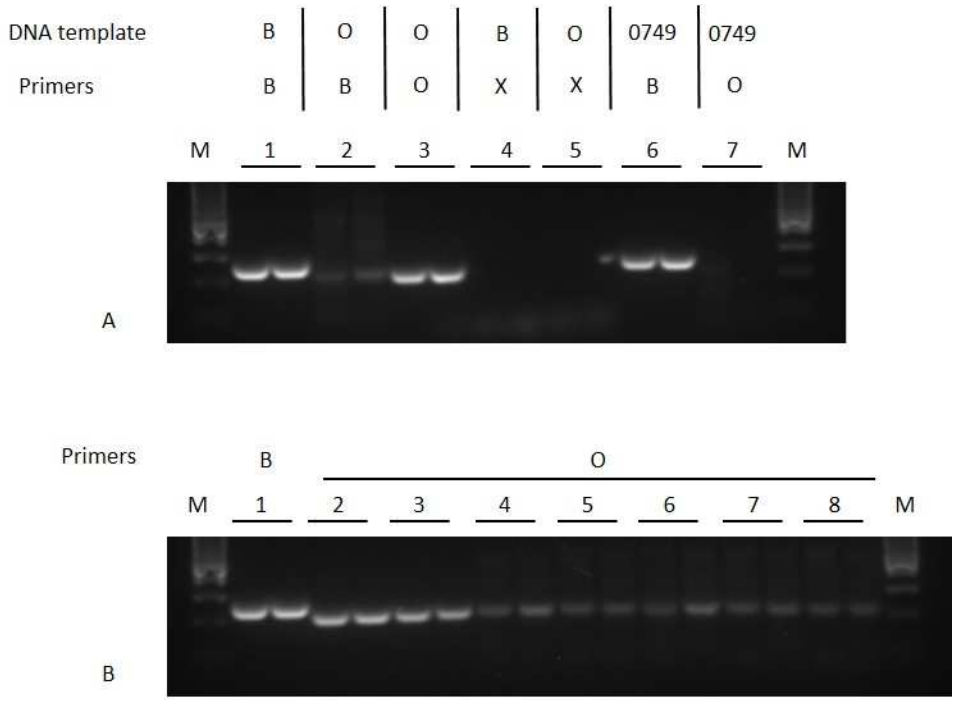


Figure 7.21. Assessment of ovine DNA content of SE1929/0749 by conventional PCR. PCR amplification of various DNA templates using both ovine and bovine primers were visualised after separation of 12.5 µl of PCR products and 3 µl of NANCY-520 in a trans-illuminator. Markers indicated by M. **A**) Bovine (B), ovine (O) or SE1929/0749 (0749) DNA template was amplified with either bovine specific (B), ovine specific (O) or without (X) primers. **B**) Dilutions series of ovine DNA into bovine DNA. Lane 1= bovine DNA and bovine primers. Lane 2= ovine DNA, ovine primers. Lanes 3-8 contained standard curve of ovine in bovine DNA dilutions and were all amplified with ovine primers (10 ng total DNA in each reaction). Lane 3=5% ovine DNA, Lane 4=1% ovine DNA, Lane 5=0.2% ovine DNA, Lane 6=0.05% ovine DNA, Lane 7= 0.025% ovine DNA, Lane 8=0.0125% ovine DNA.

To increase specificity a dilution series of ovine DNA diluted into bovine DNA was amplified in qPCR using ovine and bovine species specific primers and probe (table 2.2). The 10 fold dilution series ranged from sample containing 1% (100 pg) to 0.0001% (0.1 pg) bovine template. Amplification of 60 cycles were necessary to produce standard curves with high R^2 values (figure 7.22). Due to the high level of PCR cycles required, amplification was observed in reactions seeded with SE1929/0749 DNA (figure 7.21A), however this represented cross reactivity of ovine primers with the bovine DNA, as reactions containing bovine DNA and ovine primers also generated PCR products that produced fluorescent signal (figure 7.22B). Replicates of SE1929/0749 contained less than 0.01% ovine DNA by interpolation from the standard curve as did the bovine template with ovine primer controls (figures 7.22A, 7.22B). Together with sPMCA data, this provides evidence against scrapie contamination causing the changes in PrP^{Sc} phenotype during sPMCA.

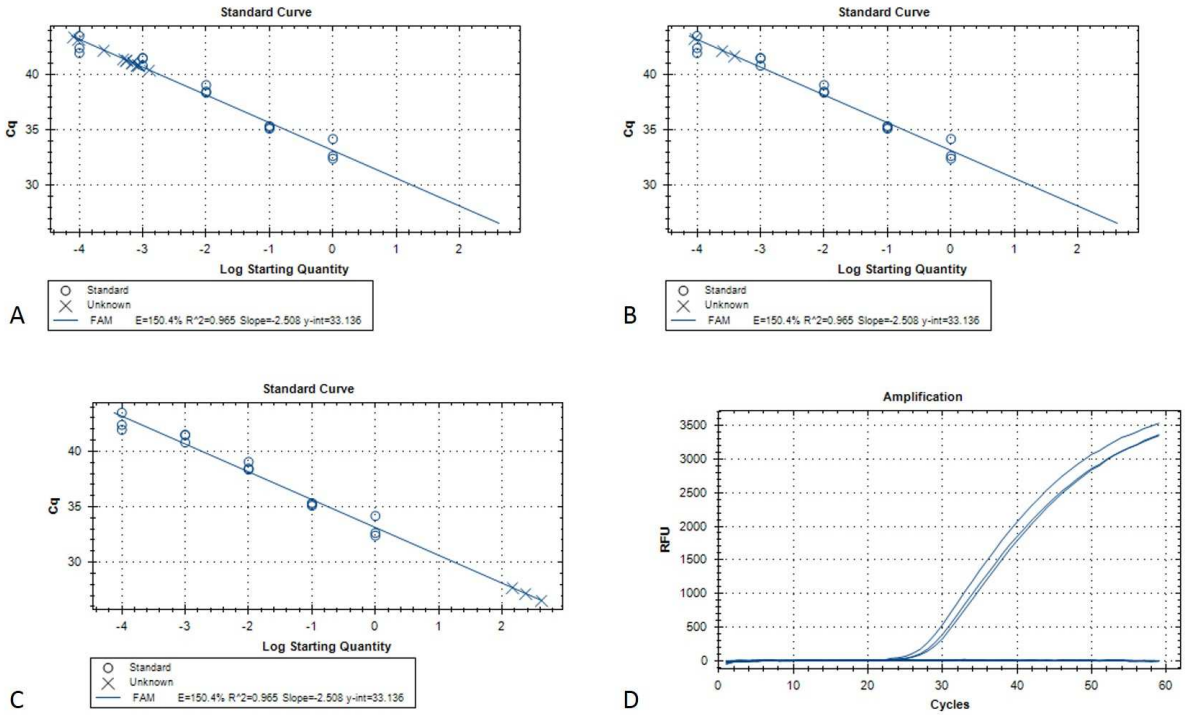


Figure 7.22. Assessment of ovine DNA content of SE1929/0749 by qPCR. Mixtures of ovine and bovine DNA containing 1%, 0.1%, 0.01%, 0.001%, 0.0001% ovine DNA content (out of 10 ng total DNA) were amplified using bovine primers and used to generate a standard curve. The relative Cq of SE1929/0749 (**A**), 10 ng of bovine DNA with ovine primers (**B**) and ovine DNA and ovine primers (**C**) are displayed. The RFU of the three positive ovine DNA with ovine primer controls is compared to three no template controls over the 60 cycles (**D**). These graphs are representative of 3 standard curves that were carried out.

7.3. Discussion

Of the initial 8 BSE samples (table 7.1) that were screened for the potential to generate PrP^{Sc} with the biochemical features of scrapie, this switch in PrP^{Sc} phenotype was observed only in SE1929/0749 when amplified in VRQ/VRQ substrates. No such switch was observed in any sample amplified in AHQ/AHQ substrate. This 1% (w/v) brain homogenate made up of 71 individual bovine BSE isolates gave rise to PrP^{Sc} with a relative high molecular weight that is associated with scrapie, and also scrapie like glycoform ratios and antibody reactivity. Forty replicate amplifications of SE1929/0749 in VRQ/VRQ substrate were positive out a total of 60, and of the 40 positive PMCA reactions 22 were had BSE-like glycoform profiles, and 18 produced more equal ratios of di-glycosylated and mono-glycosylated forms of PrP^{Sc} associated with scrapie. This phenomenon of a switch in molecular phenotype from BSE-like to scrapie-like has been described previously (Krejciova et al., 2014b) but appeared to be much more efficient than described in this study: all 4 samples analysed (one bovine BSE and three ovine BSE isolates) developed molecular profiles indicative of scrapie during

amplification in VRQ/VRQ substrate but not in ARQ/ARQ substrate. Many factors can affect the efficiency of prion amplification in PMCA, but key variables include the length and intensity of sonication periods, the time between these sonication periods and the dilution factor between rounds. Therefore the conditions documented in the literature were compared to determine whether they could increase the total efficiency and promote the molecular switching of PrP^{Sc} from more bovine BSE isolates. Using the method of Krejciova et al., two bovine BSE samples produced scrapie-like PrP^{Sc} (SE1929/0749 and BP-1) with high molecular weights, P4 antibody signal and glycoform profiles out of the 4 samples tested. Unlike the 71 bovine BSE isolates used to create SE1929/0749, the 10 single isolates of BP-1 were available to amplify in sPMCA as individual isolates. This enabled the identification of the BP-1 isolates were responsible for the formation of scrapie-like PrP^{Sc}. All 7 of the 10 isolates that amplified in VRQ/VQR substrate produced scrapie-like PrP^{Sc} verified by densitometric analysis, and a further isolate clearly generated P4 reactive products (PG01184/02), but was not designated as being a positive sPMCA amplification because of a low SHa31 signal. This means that all of the BP-1 constituents able to amplify in VRQ/VRQ substrate under these PMCA conditions could generate scrapie like PMCA products. Overall, of the 13 amplified bovine BSE isolates amplified in these conditions 9 produced scrapie like products (69%). However, to understand the true frequency of bovine BSE converting to a scrapie-like PrP^{Sc}, many more isolates should be tested under more sPMCA conditions, as this sPMCA analysed samples that had been previously demonstrated to amplify in sPMCA using VRQ/VRQ substrate and the data using sPMCA method 1 yielded only 1 sample (12.5%) that produced scrapie-like products (figure 7.1).

In either sPMCA method, scrapie-like PrP^{Sc} was only generated in VRQ/VRQ substrate, while amplification of the same isolates in AHQ/AHQ substrate only produced a scrapie-like sample. This substrate specificity for producing scrapie-like PrP^{Sc} from BSE is in accordance with the previous published work that compared VRQ/VRQ and ARQ/ARQ substrates and contributes to the authors conclusions that valine at position 136 instead of alanine in the PrP substrate for conversion appears to be crucial in generating the scrapie-like PrP^{Sc} (Krejciova et al., 2014b). The substrate specificity of the production of scrapie-like PrP^{Sc} could be explained as a result of the “transmission barrier” in the conformation selection model, in which the ability of the seed (PrP^{Sc}) to misfold PrP^{C} is dictated by the relative similarity of the two conformers, which is in turn contributed to by the primary amino acid sequence of both proteins (Collinge, 1999). AHQ/AHQ PrP^{C} was the most susceptible ovine PrP^{C} to misfolding by BSE via *in vitro* amplification compared to VRQ/VRQ which reflects *in vivo* infections in sheep and other *in vitro* amplification data (Houston et al. 2003; Krejciova et al. 2014; Priem et al. 2014). This would imply that there is greater similarity between the conformers of bovine BSE and AHQ/AHQ than VRQ/VRQ ovine PrP^{C}. It is possible that not only does valine at position 136 make prion

misfolding less efficient, it enables an alternate PrP^{Sc} misfolding route that produces a scrapie-like conformer. It is also important to note that sheep with the VRQ/VRQ genotype are most susceptible to scrapie infection, which may imply that the valine at position 136 of PrP^{C} is a key determinant in PrP^{C} structural preference for a scrapie-like PrP^{Sc} conformer (Hunter, 2003; Hunter et al., 1996).

The PrP^{Sc} generated through the amplification of SE1929/0749 in the primary PMCA was characterised as classical BSE or scrapie-like, through glycoform profiling and by using core and N-terminal antibodies. All of the PrP^{Sc} produced in AHQ/AHQ substrate had a molecular weight typical of BSE, did not react strongly with N-terminal antibody P4 and had di-glycosylated and mono-glycosylated isoform content typical of BSE ($BSE_{A1:1}$). Characterisation of the products from VRQ/VRQ amplification illustrated a range of more distinct PrP^{Sc} types. The glycoform profiles of the products were placed into three groups, those with profiles that clustered with 4 other bovine BSE isolates, those that clustered within scrapie isolates and those PMCA products that fell between the two groups; BSE ($BSE_{1:1}$) scrapie like ($Sc_{1:1}$) and intermediate ($Int_{1:1}$) respectively. Both the $Sc_{1:1}$ and the $Int_{1:1}$ products had scrapie-like molecular weights and reacted with the core antibody P4, which specifically recognises an epitope in scrapie and not BSE PrP^{Sc}, yet the $BSE_{1:1}$ PrP^{Sc} molecular weights were standard for BSE and no P4 signal was detected.

The different populations of PrP^{Sc} generated in the primary sPMCA reactions were then used to seed a secondary PMCA (figure 7.8) in both VRQ/VRQ and AHQ/AHQ substrates. Amplification of $BSE_{A1:1}$ in either substrate generated typical BSE products. However, all three of the VRQ/VRQ derived primary PMCA populations ($Sc_{1:1}$, $Int_{1:1}$ and $BSE_{1:1}$), when amplified for a further 5 rounds in VRQ/VRQ substrate produced high molecular weight products with glycosylation profiles identical to scrapie and reactivity with P4 antibody. The only difference between the $Sc_{2:1}$, $Sc_{2:2}$ and $Sc_{2:3}$ populations is that when amplified in the alternating substrate sPMCA assay, no products are detected when seeded with the $Sc_{2:1}$ but PrP^{Sc} with a scrapie profile is detected in $Sc_{2:2}$ and $Sc_{2:3}$, this observation in itself is noteworthy. This is in contrast to previously reported data for this sPMCA assay in this thesis in which scrapie did not produce SHa31 reactive sPMCA products (chapters 5 and 6). However in published data 1 out of 108 scrapie isolates tested for the ability to seed sPMCA reactions in this assay generated PrP^{Sc} which was SHa31 positive but displayed a scrapie like SHa31:P4 ratio, this isolate was ARH/VRQ *PRNP* genotype (Gough et al., 2014). This difference may be indicative of the strain selection and mutation processes which proceeds during the *in vitro* amplification of PrP^{Sc} and *in vivo* disease progression of TSEs. One explanation of TSE strains commonly referred to as the cloud hypothesis, stipulates that strains are made up of populations, or clouds, of PrP^{Sc} with different conformations (Collinge and Clarke, 2007). The amplification conditions *in vitro* or different host associated factors *in vivo* select for the most kinetically favourable member of the cloud, in these conditions, and this PrP^{Sc} becomes the

predominant PrP^{Sc} conformer. If the conditions are changed such that the formation of another version of PrP^{Sc} becomes the most favourable then this conformer will become dominant, and if the properties of this second conformer differ from the first then the effect of strain mutation may be observed, as may be the case with the BSE seeds that produce the scrapie like PrP^{Sc} products. However, for the cloud hypothesis to be applicable to the secondary PMCA products and account for the difference between $Sc_{2:1}$ and the other scrapie like products ($Sc_{2:2}$ and $Sc_{2:3}$), the predominant conformer must switch from BSE to two distinct scrapie-like conformers, one which can amplify in the alternating sPMCA assay and one that cannot. However, an alternate strain selection hypothesis exists (sometimes referred to as the deformed template hypothesis) in which instead of a cloud of different conformers present in the seed, a single conformation of PrP^{Sc} exists, and when the conditions for misfolding change this acts to drive changes in conformation of PrP^{Sc} in which misfolding is more energetically favourable (Collinge and Clarke, 2007; Makarava and Baskakov, 2013). These two theories are not mutually exclusive and amplification of bovine BSE in ovine VRQ/VRQ substrate could generate a cloud of new PrP^{Sc} conformations, one of which would then become dominant due having the most thermodynamically favourable misfolding kinetics. With VRQ/VRQ substrate, on some occasions this could be a BSE-like product and on others it could be a scrapie-like product. The latter could be further divergent with some products having no conformers that can be replicated in the alternating substrate reactions and others containing conformers that can replicate under these conditions.

Further evidence of strain selection is displayed on amplification of the $BSE_{1:1}$ population. Amplification of this population in AHQ/AHQ generated BSE-like PrP^{Sc} in contrast to when amplification was in VRQ/VRQ substrates which yielded a scrapie-like product. Amplification of the $Int_{1:1}$ and $Sc_{1:1}$ populations in AHQ/AHQ also yielded BSE like products, aside from the intermediate glycoform profiles of the replicates, this indicates that all of the primary PMCA populations retain the potential to form BSE like sPMCA products, in terms of the cloud hypothesis, BSE PrP^{Sc} was still present.

Using the alternating substrate sPMCA assay, BSE was not found to be present in either $Sc_{2:1}$ or $Int_{2:1}$ populations, given the specificity and sensitivity of this assay (both specificity and sensitivity of 100% when seeded with artificial mixes containing 0.1% bovine and 99.9% ovine brain material) it is likely that BSE conformers are no longer present in either of the secondary PMCA products and therefore fully out competed by the scrapie-like conformers during VRQ/VRQ sPMCA (Simmons et al., 2015). Considering that probing $Int_{2:1}$ with primary core and N-terminal antibodies (SHa31 and P4) produces a binding profile that is BSE-like, the inability of the sPMCA assay to amplify BSE prions unexpected.

This may be indicative that the molecular switch occurring is a complex multistage process and $Int_{2:1}$ lies between classical BSE and $Sc_{2:2}$ PrP^{Sc}.

We also aimed to use conformational stability assays to further characterise and group the secondary sPMCA products based on the conformation of PrP^{Sc} present in the products. To enable us to make inferences for the CSAs a range of scrapie and ovine BSE isolates, some of which had been subjected to sPMCA were tested by CSA. However there appeared to be no meaningful differences between the scrapie and BSE isolates, and the scrapie and BSE isolates did not cluster. This finding does not reflect the CSA profiles reported in the literature where bovine BSE isolates are far more resistant to denaturation with higher $Gdn_{[50]}$ values compared to scrapie (Masujin et al., 2008; Pirisinu et al., 2011; Vrentas et al., 2013). However the $Gdn_{[50]}$ values were far more representative of ovine BSE and scrapie that have previously been documented in the literature. Bovine BSE amplified in sPMCA was found to be significantly more susceptible to degradation than PrP^{Sc} from brain tissues however the same relationship was not identified in scrapie isolates. This implies that the conformation of BSE PrP^{Sc} being selected for that could replicate efficiently in ovine VRQ/VRQ substrate, while a difference between sPMCA and brain derived PrP^{Sc} was not observed in scrapie (derived from VRQ/VRQ animal) because the scrapie PrP^{Sc} could already replicate efficiently and was not under the same thermodynamic selection drivers. Despite the difficulties in reproducing CSA results for scrapie and BSE that reflected what has already been documented in the literature, the SE1929/0749 that were subjected to denaturation at 3.0M Gdn-HCl fell into two groups with statistical significance by one-way ANOVA and Tukeys multiple comparison. Those PMCA products with scrapie-like PrP^{Sc} were significantly more sensitive to denaturation than those with typical BSE-like PrP^{Sc}, and comparisons of the two previously have always found scrapie PrP^{Sc} more sensitive to ovine BSE PrP^{Sc} (Masujin et al., 2008; Pirisinu et al., 2011; Vrentas et al., 2013).

The generation of a scrapie-like PrP^{Sc} derived from BSE has important implications for surveillance in small ruminants. As the zoonotic potential of BSE was realised with the discovery of vCJD, and the cause of the BSE in cattle was infected meat and bone meal which was not only used as feed for cattle but also additionally for small ruminants (Will et al., 1996). This raised two important questions, could the sheep at the time of the BSE epidemic have also been infected with BSE, and if so would ovine BSE still pose a zoonotic risks to humans through infected food. The threat of another BSE epidemic in sheep led to systematic and routine testing of sheep displaying TSE symptoms, yet despite intense surveillance no sheep have been described with natural cases of BSE, and only two goats have been identified with BSE field-infections (Eloit et al., 2005; Jeffrey et al., 2006b; Stack et al., 2006). sPMCA can be used to model animal passage much more quickly and requiring much less resources than bioassay equivalents, therefore the generation of scrapie-like products during sPMCA raises the

possibility that if BSE did infect the UK sheep flock, it may have changed phenotype to resemble scrapie and if this adaptation occurred before or during the introduction of strain typing tests, BSE in small ruminants may have gone unnoticed (Krejciova et al. 2014). To model whether or not BSE with a scrapie-like phenotype was still a zoonotic threat to humans Krejciova *et al*. seeded human brain substrate with scrapie like PMCA products and found that the ability to replicate in this substrate in a single round of PMCA had been lost, compared to the seed which could misfold human substrate that was homozygous for methionine at position 129. The authors did not declare if any further rounds of sPMCA were conducted, but the generation of scrapie-like products from BSE took a minimum of 6 rounds of sPMCA, if the process were reversible it may also require a similar number of rounds of sPMCA therefore the zoonotic potential of scrapie-like BSE products may still be unknown.

An additional aspect of the molecular plasticity of bovine BSE is that the intermediate population $Int_{2:1}$ produced upon passage of the spike through two sPMCA experiments using VRQ/VRQ substrate has similarities to the scrapie strain CH1641. CH1641 is classed as a BSE-like scrapie isolate in terms of its biochemical signatures, both CH1641 and $Int_{2:1}$ and classical BSE isolates do not possess the epitope for P4 antibody (Stack et al., 2002). Yet in the alternating substrate sPMCA assay, $Int_{2.1}$ (table 7.4) and CH1641 do not contain any BSE PrP^{Sc} (Gough et al., 2014). CH1641 possesses an intermediate glycoform between scrapie and BSE, and is more susceptible to degradation by Gdn-HCl than BSE isolates, however $Int_{2:1}$ also has an intermediate glycoform profile, though we were able to differentiate between the two using conformation stability assays (Pirisinu et al., 2011; Stack et al., 2002). CH1641 has been shown to possess a CTF14 fragment which is not present in BSE that can be detected by SAF84 antibody, we applied this assay to CH1641 and $Int_{2:1}$ but the CTF14 fragment was not detected in any samples including the four CH1641 isolates (Nicot et al. 2010). This indicates that this CTF14 assay is difficult to reproduce across laboratories and could not be used here to further characterise $Int_{2.1}$. The gold standard assay used for strain typing TSE infections is rodent bioassay and transgenic mouse bioassay. Transgenic mouse bioassay can differentiate between strains of scrapie, including BSE-like CH1641 and BSE using incubation periods, immunohistochemistry and neuropathology of infected animals (Bencsik et al. 2011; Scott et al. 2005). Bioassays are currently being planned in which a range of transgenic mice expressing ovine, bovine and human will be inoculated with a range of sPMCA products after 10 rounds of amplification, which will give further indication of the infectious threat to humans and animals of scrapie-like BSE.

The results of this study offer further evidence for the formation of scrapie-like PrP^{Sc} generated when BSE misfolds PrP^{C} from the VRQ/VRQ genotype and is in agreement with already published data (Krejciova et al., 2014b). Using two sPMCA methods BSE PrP^{Sc} amplified in VRQ/VRQ substrate has been generated different populations of PrP^{Sc} that share molecular characteristics with scrapie. These

populations can resemble scrapie in terms of molecular weight, antibody reactivity and proportion of glycosylated protein, yet the extent to the molecular similarity can vary with different populations and the reason for this array of molecular phenotypes remains unclear. Furthermore using assays specifically designed to amplify BSE PrP^{Sc} we have failed to detect the presence of BSE, despite the presence of PrP^{Sc} which has a BSE antibody reactivity profile ($Int_{2:1}$). The further characterisation applied to the various PrP^{Sc} types generated in sPMCA has detected several variations in scrapie-like and BSE-like PrP^{Sc}, one of which appears similar to CH1641 scrapie. The generation of these distinct PrP^{Sc} types likely results from a cloud of PrP^{Sc} that is adapting to the replication conditions to form the most kinetically favourable PrP^{Sc} molecules, which may prove valuable in further understanding *in vivo* infections. If sPMCA acts as a representative model for *in vivo* infections, in some instances the molecular presentation of ovine BSE PrP^{Sc} may be more complicated than originally suspected, and as a result particular care must be taken in biochemical analysis of PrP^{Sc} from suspect ovine TSE cases.

Chapter 8. Conclusions and recommendations for further study

The results of this thesis make a significant contribution to the understanding of prion diseases through the use of the *in vitro* amplification technique sPMCA. The use of recombinant hamster PrP was employed to successfully amplify scrapie PrP^{Sc} from a range of different *PRNP* genotypes however the low specificity of this assay was the limiting factor in providing a universal substrate to amplify scrapie strains. sPMCA was used to successfully quantify BSE, using brain homogenate as the PrP^{C} substrate, however a correlation was not observed between replication efficiency and infectious titre in bioassay. Atypical L-type and H-type BSE have successfully been amplified and the sPMCA products characterised, the amplification of H-type in sPMCA has not been previously described. A method of selectively amplifying BSE PrP^{Sc} in the presence of excess scrapie has also been shown to have a sensitivity greater than previously described methods. This assay was then also applied to ovine scrapie and BSE co-infections of sheep. The assay had greater sensitivity than conventional tests used and described for the first time the presence of ovine scrapie and BSE co-infections. High risk sheep genotypes were identified and BSE was detected after inoculation to an animal with a field scrapie infection. Finally, bovine BSE after amplification in ovine substrates has been demonstrated to change the molecular phenotype of BSE to resemble scrapie PrP^{Sc}, in terms of molecular weight, glycoform ratio, antibody reactivity and BSE PrP^{Sc} presence determined by a novel sPMCA assay.

The outcomes of this thesis may also be framed within the view of *in vitro* evolution of PrP^{Sc} conformers through replication in sPMCA. The process of the *in vitro* evolution of prions is an example of molecular evolution and is conceptually analogous to genetic molecular evolution. In simple terms, mutations create diversity in alleles for genes, which are passed on to subsequent generations. Natural selection acts upon this population of alleles and selects for those with the greatest fitness. In this metaphor different conformations of prions are analogous to genetic alleles and each prion strain contains many different conformers, but one or several dominant isoforms, isolate according to the cloud hypothesis which can be selected for (Collinge and Clarke, 2007). During amplification in sPMCA the PrP^{Sc} conformers with the most favourable thermodynamic reactions for misfolding PrP^{C} are selected for. Changes or mutations in conformers which make up the cloud are thought to occur when the environment for misfolding changes. Within sPMCA these mutations would most likely proceed through changing of the genotype of PrP for replication from the previous substrate, whether that was *in vivo* or *in vitro*. Other factors such as temperature at which misfolding takes places and the frequency, power and duration of sonication could skew the thermodynamic misfolding reactions to favour another conformer.

The observed generation of protease resistant PrP from recombinant substrate in sPMCA highlights the ability of the sPMCA environment to influence the structure and cause mutation in PrP^{Sc}

conformers. Seeding of recombinant PrP as substrate with both healthy brain material and reactions that contained substrate alone generated PrP^{Sc} conformers. In this case it is possible that the exposure of the recombinant PrP to sonication induced the changes in the recombinant PrP structure and the production of protease resistant recombinant proteins.

Amplification of H-type BSE in sPMCA developed products that resembled the seed in terms of molecular weight but were far more sensitive to PK digestion. Mutation of the H-type BSE to conformers with low PK resistance could have occurred if unknown genotypic differences in the PrP^{C} between of the bovine substrate for sPMCA and the PrP^{C} of the effected animal. Alternatively this conformer may already have existed in the cloud of PrP^{Sc} conformers and was selected for by the reaction kinetics of sPMCA. For the efficient amplification of L-type BSE Teflon beads were required in the sPMCA. The addition of these beads may have acted upon the reaction kinetics of the misfolding process to distribute energy from sonication process to enable a mutation of a conformer that could replicate. Conversely the Teflon beads may have changed the selection on the cloud of conformers already present in the L-type BSE isolate. These two scenarios are not mutually exclusive and the addition of Teflon beads could simultaneously have caused the generation of a new conformer from those existing in the cloud and changed the reaction kinetics to select for that conformer.

The alternating sPMCA technique that could selectively amplify BSE in the presence of excess scrapie prions demonstrates the complicated nature of selection and mutation of conformers with two independent clouds of conformers competing for replication. Alternating the substrate between two sheep genotypes (AHQ/AHQ and VRQ/VRQ) at every round of sPMCA created different selection pressures in these rounds. This selection may have favoured the amplification of BSE as the cloud of conformers constituting BSE could be more diverse. Alternatively the BSE conformers may be more malleable to the selection pressures, with the change in PrP^{C} genotype creating a larger or more diverse group of BSE conformers compared to scrapie.

The ability of classical BSE to display molecular phenotypes of scrapie in certain amplification conditions can also be explained through the *in vitro* evolution of the prions. The switch in the molecular characteristics could be achieved only in ovine substrate of VRQ/VRQ genotype. Therefor the VRQ/VRQ PrP^{C} facilitated the mutation of BSE to scrapie like conformers or was able to select for scrapie like conformers that already existed as a small part of the BSE population. Furthermore the scrapie like BSE conformers could be selected for by changing the sPMCA conditions to match those previously published. Reducing the sonication time and dilution of sPMCA products between rounds, while extending the number of cycles within a single sPMCA round scrapie like conformers were observed in BSE isolates which were not previously described. Therefore this particular sPMCA

scenario was much more efficient at causing mutations to scrapie like conformers and then selecting for these.

8.1. Replacement of bioassays that quantify sPMCA products using *in vitro* amplification techniques

To develop *in vitro* quantification assays which could replace animal bioassays the first objective was to successfully amplify a range of scrapie isolates from sheep with distinct genotypes using recombinant hamster PrP as substrate for sPMCA. Amplification of scrapie was achieved with 100% sensitivity for all scrapie isolates that were assayed. The scrapie isolates that were amplified were from a wide range of *PRNP* genotypes, which is a major advantage over animal bioassays and sPMCA with PrP^{C} substrate derived from brain homogenates which often have to be matched based on *PRNP* genotypes in order to achieve successful disease progression or amplification. Therefore in theory the use of recombinant sPMCA could be applied to standardised *in vitro* quantification of infectivity of scrapie isolates by qPMCA. However the limitation of this substrate was the high rate of PK-resistant PrP that was derived from only substrate or when seeded with healthy brain homogenates. Despite additives and modifications to the sPMCA method, this non-specific formation of PK-resistant PrP could not be eliminated. Spontaneous formation of PK-resistant products from recombinant has been documented before in sPMCA, and can even be induced solely by shaking recombinant PrP (Atarashi et al., 2011; Ladner-Keay et al., 2014). To address the limitations of the recombinant sPMCA, many additional changes to the sPMCA set up could be recommended. False positives in sPMCA using recombinant hamster substrate have been reduced by addition of Triton X-100, therefore this could be included in the sPMCA reactions described here along with other detergents, furthermore *de novo* PK-resistant PrP was differentiated from PrP^{Sc} seeded by differential reactivity with antibodies (Atarashi et al., 2007). Here, sPMCA products were only probed with SHa31 primary antibody, a panel of antibodies could be applied to seeded and *de novo* PK-resistant PK products. Several factors are known to induce the formation of PK-resistant PrP fibrils which include divalent cations, nucleic acids, phospholipids and pH, varying these in sPMCA reactions may reduce the levels of false positives observed (Baskakov et al., 2002; Bocharova et al., 2005; Deleault et al., 2003; Wang et al., 2007). Teflon beads in sPMCA reactions have also been shown to increase specificity of amplification, introduction of these may also be advantageous in reducing PK-resistant PrP (Gonzalez-Montalban et al., 2011). Due to the misfolding of recombinant PrP from shaking alone, decreasing the sonication time to produce less agitation of the recombinant proteins may also reduce *de novo* PK-resistant PrP formation rate. Additionally recombinant PrP protein produced in the baculovirus expression system in insect cells more closely resemble mammalian PrP, due to eukaryotic post-translational

modifications of the proteins, because of this these proteins may be less likely to spontaneously form PK-resistant PrP, and as such they could also be trialled as a substrate (Faburay et al., 2014). There was a higher rate of *de novo* PK-resistant PrP in the reactions seeded with healthy brain homogenates, which could be due to the co-factors present in the brain homogenates, to reduce the level of these co-factors in the seeds the PrP^{Sc} could be purified prior to use as a seed.

Bank vole substrate is perhaps a more promising candidate for a universal substrate than recombinant hamster PrP. In RT-QuIC it has been used to amplify at least 29 TSE strains, including major human and animal prion PrP^{Sc} (Orrú et al. 2015; Masujin et al. 2016). Therefore recombinant bank vole substrate could also be implemented as a substrate in qPMCA to test if it is as sensitive as in RT-QuIC and if it is any more specific than recombinant hamster PrP.

Developing a recombinant sPMCA method was originally designed to aid a qPMCA technique that would be able to quantify a broad range of PrP^{Sc} types of ovine scrapie isolates from different *PRNP* backgrounds. However as the recombinant substrate was not suitable, PrP^{C} in brain homogenates were used to develop the qPMCA and amplification was focused on BSE, to prevent scrapie strain effects on amplification efficiency. The qPMCA was able to estimate the seed content of BSE amplified in ovine AHQ/AHQ substrates respectively with an error of 40% and 1% depending on amount of brain content used to seed reactions. Quantification of PrP^{Sc} load from various sources has a range of applications, it allows greater understanding of disease progression by analysing PrP^{Sc} deposition in different tissues, enable identification of subclinical carriers and assessment of a range of decontamination techniques. The qPMCA in this study was not as sensitive as previously described techniques methodologies (Samuel E. Saunders et al., 2011). Furthermore the sample sizes for BSE were low, amplification of a greater number of independent repeats are needed to validate the experimental findings. None of the published studies attempt to address the reproducibility of this techniques between BSE isolates. For the potential of qPMCA to be realised assessing the individual variation in amplification efficiency between BSE isolates which have been assayed by bioassay is essential. However data indicated that in this qPMCA method there was not a strong correlation between amplification efficiency and infectivity in mouse bioassays. Therefore this qPMCA currently could not be used to estimate the relative infectivity in animal bioassay of an unknown sample using a reference BSE isolate of a defined titre as a standard for replication efficiency. The PrP^{Sc} of different classical BSE isolates is thought to be conserved, therefore the difference in replication efficiency may be due to host co-factors in the infected bovine brain homogenates. To test this theory precipitation of PrP^{Sc} from bovine brain homogenates prior to qPMCA could be trialled to remove host specific co-factors to assess if a stronger correlation between replication efficiency and infectivity could be established between BSE isolates.

8.2. *In vitro* amplification and characterisation of atypical BSE

sPMCA conditions have been developed to amplify both atypical H-type and L-type BSE. Amplification of H-type BSE, which has not been described before was particularly efficient, sPMCA was able to detect seeds down to 10 fg in one isolate. The H-type BSE sPMCA products could be differentiated from those seeded with classical BSE in a number of ways including by molecular weight, PK resistance and antibody reactivity. Previously only L-type amplification has been demonstrated in sPMCA, which required additional co-factors for amplification such as poly-anions and L-arginine ethylester, which were not included in the sPMCA described here (Murayama et al., 2016). *In vitro* amplification methods are of interest because of their potential to lead to novel diagnostic approaches and surveillance techniques which could help prevent further BSE epidemics in the future. Surveillance of atypical BSEs is of particular importance because animals infected naturally tend to be old animals that are asymptomatic carriers and as such would not be identified by routine surveillance (Brown et al., 2006). Furthermore, detection methods and further study of atypical BSE to prevent human consumption is of high importance as experimental challenges of humanised mice and macaques indicate that H-type and L-type BSE pose more of a zoonotic threat than classical BSE (Béringue et al., 2008; Comoy et al., 2008; Kong et al., 2008). As atypical BSE may be sporadic in nature, the development of techniques that can be used to further study atypical BSE may provide important insights, into human sporadic prion disease. Amplification of atypical BSE diseases may be used to assess models of human sporadic TSE disease and act as an *in vitro* model for replication of prion misfolding which could identify important co-factors or conditions that induce sporadic TSE disease.

Both atypical BSEs have also been amplified using RT-QuIC, however the main advantage of sPMCA is that it can generate PrP^{Sc} that can be further studied, and resembles more closely the seed, retaining infectivity (Masujin et al. 2016;Orrú et al. 2015b). To establish sPMCA based surveillance technique able to distinguish classical BSE and atypical H-type and L-type BSE isolates the sensitivity of detection of L-type BSE by sPMCA would need to be improved. Currently the detection limit for L-type BSE in this sPMCA method is 100 µg, and this only seeds amplification in ~50% of replicates indicating that this is just on the limit of detection. Were the sensitivity of amplification to be improved, perhaps by the addition of aggregating agents such as polyanionic nucleic acids or nitrocellulose particles, an important sPMCA based surveillance technique able to distinguish classical and atypical BSEs could be designed and implemented.

8.3. Development and evaluation of a serial PMCA assay to specifically identify BSE in the presence of excess amounts of scrapie.

A modification of sPMCA has been described in which the brain homogenate substrate is alternated between two different ovine *PRNP* genotypes, AHQ/AHQ and VRQ/VRQ, at each round. The alternation of the substrate selectively amplifies BSE prions, even in the presence of excess amounts of scrapie prion. Using this technique BSE was identified at 0.1% in the seed containing 99.9% scrapie with 100% sensitivity and specificity in 4 different scrapie isolates tested in a double blind trial. In one isolate, BSE was amplified at 0.01% which was the lowest dilution tested. The samples that were amplified in this assay were also tested at the APHA by western blots, ELISA and bioassay (Simmons et al., 2015). sPMCA was by far the most sensitive of any *in vitro* assay, the highest sensitivity of western blots was for samples containing 75% BSE and ELISA was 10% BSE. The bioassay could detect 1% when BSE was diluted into classical VRQ/VRQ or AHQ/AHQ scrapie (which was the lowest concentration tested), BSE diluted in atypical scrapie was only subjected to bioassay in mice at 1% BSE content and the bioassay failed to detect the presence of BSE in the inoculum. BSE was also diluted into classical 1-4-7 ARQ/ARQ scrapie, and could only be detected when BSE was present in the inoculum at 25%. In this instance the sensitivity of the sPMCA was greater than that of the bioassay and was recommended for inclusion on the European Union reference laboratory statutory testing panel. This assay represents an important step forward in answering questions regarding the lack of BSE cases in small ruminants; only 2 cases, both in goats have ever been reported. During and prior to the BSE epidemic sheep and goats were fed MBM, potentially infected with BSE, despite being experimentally infected by BSE natural cases have only occurred in 2 goats, the reason for the very low transmission is unknown. One possible theory is that the endemic scrapie infection in the UK sheep population at the time might have prevented detection of BSE. This assay enables the analysis of BSE and scrapie co-infections and would be able to determine if very low levels of BSE could be masked by much larger scrapie signals in conventional surveillance tests. To more accurately define the limits of this assay, rather than using crude mixtures of brain homogenates with different concentrations of PrP^{Sc}, purified PrP^{Sc} (potentially by NaPTA) concentrations could be estimated by comparison with recombinant PrP. Alternatively the signal generated from the brain homogenates following western blotting could be used to estimate the relative PrP^{Sc} content of each brain homogenate, to produce mixtures that were more accurately defined. To apply this test further, the assay was applied to *in vivo* mixed infections.

8.4. *In vitro* detection of BSE prions in ovine scrapie and BSE co-infections

Previously, alternating the substrate used for sPMCA amplification between AHQ/AHQ and VRQ/VRQ *PRNP* genotypes proved to selectively amplify BSE when seeds contained only 0.1% BSE and 99.9% scrapie with 100% specificity and sensitivity. This assay was applied to the brain, spleen and prescapular lymph nodes of ARQ/ARQ and VRQ/ARQ sheep infected with BSE and scrapie at the same time or with a delayed BSE infection by three months. BSE could not be detected in VRQ/ARQ sheep that received the scrapie and BSE on the same day or with a delayed BSE infection, while BSE only infections could be detected. This implies that in these sheep scrapie out-competed BSE for PrP^{C} substrate, and BSE disease progression did not occur. For ARQ/VRQ sheep, BSE was detected in all brain but not all spleen and lymph node samples when sheep were administered with BSE and scrapie, BSE was found less often in samples with a delayed BSE infection. In one case an animal was BSE free, one animal had BSE in the brain but not the periphery and in another the converse was true. This data paints a very complicated picture of PrP^{Sc} deposition during disease progression in scrapie and BSE co-infections. For the first time we have demonstrated that ovine scrapie and BSE mixed infections can occur, and furthermore sPMCA can detect BSE in low quantities in the brains and peripheries of animals only found to be infected by scrapie using conventional methods.

The same assay was also applied to sheep from a flock with an endemic scrapie infection, all animals of VRQ/VRQ genotype develop scrapie at around 8 months of age. These animals were injected in the hind leg with BSE at 8 months of age, after scrapie infection was very likely to have already become established. BSE was detected in the brain of one of these animals, which was not detected by conventional testing.

This assay has great potential for the surveillance of sheep populations for possible low level BSE infections including when scrapie infections are present and could be applied to suspect scrapie cases where PrP^{Sc} profiles or pathology are not like classical scrapie when measured by other assays (Beck et al., 2012; Bencsik and Baron, 2011; Gretzschel et al., 2005). Furthermore, the assay has identified that those sheep with an ARQ allele for *PRNP* are at higher risk of a BSE/scrapie co-infection than VRQ alleles.

The development of this assay and discovery of BSE and scrapie co-infections may have important implications regarding human prion diseases in the future. Demonstrating that a subclinical BSE infection can persist within an animal with a dominant scrapie infection, highlights that multiple prion diseases may exist in the same person as separate entities, and that these may localise to different tissues. This knowledge may aid the identification of more mixed TSE diseases of humans.

To further characterise mixed infections, larger numbers of replicates of sheep could be inoculated including sheep with ARR and AHQ alleles. Though expensive this would allow full identification of the susceptibility of sheep to BSE in mixed infections. Furthermore, analysis of more tissue types would enable identification of 'at-risk' tissues for BSE contamination in mixed infections. As well as hind leg injection, sheep could be dosed orally to produce a more natural disease progression, though infectivity rates and PrP^{Sc} tissue deposition may be extremely low.

8.5. Identification and characterisation of bovine BSE which upon *in vitro* amplification demonstrates biochemical characteristics of scrapie in a genotype dependent manner.

In vitro amplification of BSE in VRQ/VRQ ovine substrates has produced BSE that shares molecular characteristics associated with scrapie. The likelihood of this being a scrapie contaminant is extremely low, no ovine DNA material can be found in the samples tested and this molecular switch has been demonstrated in 9 separate BSE isolates in this thesis, and has been demonstrated in 4 isolates by independent researchers (Krejciova et al., 2014a).

BSE amplified in VRQ/VRQ substrate after 10 rounds of amplification could display scrapie-like molecular characteristics in terms of glycoform profile, molecular weight, antibody reactivity and did not contain BSE as assayed by the alternating substrate sPMCA designed to amplify ovine BSE in excess quantities of scrapie. Amplification of the same BSE isolate in AHQ/AHQ substrate for 10 rounds did not produce scrapie-like PrP^{Sc} in any of the assays tested. After 10 rounds of amplification in VRQ/VRQ some replicates produced a population of PrP^{Sc} that had an intermediate glycoform ratio, had P4 reactivity and molecular weight similar to BSE but did not contain BSE prions as tested by the alternating substrate sPMCA assay, a molecular phenotype resembling CH1641 scrapie.

Amplification of prions in sPMCA serves as a model for *in vivo* disease progression, therefore the discovery of BSE which can switch molecular phenotype to resemble scrapie during amplification in VRQ/VRQ is worrying as it implies that BSE infection in sheep if allowed to replicate in the population could produce BSE-derived PrP^{Sc} resembling scrapie. PrP^{Sc} molecular phenotype is the definitive marker for TSE infection so such plasticity if found to occur on a large scale may hamper TSE identification. This finding also raises questions about the zoonotic potential of BSE with a scrapie-like phenotype, previously scrapie like BSE failed to convert human PrP^{C} in sPMCA while BSE retained seeding ability in this substrate, indicating a lower zoonotic potential of scrapie-like BSE PrP^{Sc}(Krejciova et al., 2014a). The switch occurred solely in PrP^{c} substrate of VRQ/VRQ genotype indicating this is an at risk genotype for this phenomenon.

With respect to human prion disease the documentation of this phenomena opens the possibility of a changing infection with disease progression. For example the infection of an individual with vCJD may induce another human prion disease, such as sCJD or FFI, due to the molecular evolution of the prion conformers during disease progression.

Recommendations for further study would be to focus on triggers for the molecular switch, and if this is a property that is present in every BSE isolate. The two sPMCA conditions tested here had different efficacies in switching the molecular phenotype and not all BSE switched to a scrapie-like PrP^{Sc}. The optimum conditions that cause BSE switching could be more accurately defined, varying sonication time and trialling sPMCA additives for instance. Following optimisation this could be applied to a large BSE cohort to find the rate at which the molecular switching of BSE occurs. Finding the optimum conditions for scrapie-like PrP^{Sc} generation may also indicate if the cause of the phenomenon has physiological relevance. The question of the zoonotic potential should be addressed. Previous studies have seeded human substrate with scrapie-like BSE in sPMCA which failed to amplify indicating a transmission barrier to human infection is present, replication of this experiment with scrapie-like sPMCA products generated in this study would give further indication of the zoonotic potential in independently generated PrP^{Sc}. In addition to sPMCA modelling transmission barrier, testing a range of PrP^{Sc} populations which have been amplified for 10 rounds in sPMCA in different rodent bioassays to determine if infection can occur in transgenic mice expressing ovine, bovine and human *PRNP*, and if the infections can be differentiated from scrapie and BSE infections would enable proper assessment of the threat posed by scrapie-like BSE. Finally the difference between BSE isolates which caused some to produce scrapie-like PrP^{Sc} while others produced BSE PrP^{Sc} should be addressed. Classical BSE is thought to have a conserved PrP^{Sc} conformer between isolates, in effect only one strain exists although evidence of sub populations containing PrP^{Sc} with high molecular weight and scrapie like antibody has previously been described (Yull et al., 2006). If this is true than it is possible that host factors could be responsible in the brain homogenate for inhibiting this 'molecular switch'. To test this theory, BSE PrP^{Sc} with the potential to form scrapie-like conformers could be precipitated and spiked into brain homogenates that only form BSE PrP^{Sc} or do not replicate, prior to sPMCA amplification.

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Appendix

Table 1 Healthy brain homogenates

ID	TSE	Species	Genotype	Breed	Source
PG0143/12	Healthy	Ovine	VRQ/VRQ		APHA Weybridge
PG0210/12	Healthy	Ovine	AHQ/AHQ		APHA Weybridge
PG1562/10	Healthy	Ovine	ARQ/ARQ	Suffolk	
PG0209/12	Healthy	Ovine	AHQ/AHQ		APHA Weybridge
PG1384/10	Healthy	Ovine	VRQ/VRQ		
PG1418/08	Healthy	Ovine	ARQ/ARQ		
PG1409/08	Healthy	Ovine	ARR/ARR		
PG1410/08	Healthy	Ovine	ARR/ARR		
DK033 4070 2486	Healthy	Ovine	VRQ/VRQ	Holstein	
PG0649/09	Healthy	Ovine	VRQ/VRQ		
PG0664/09	Healthy	Ovine	VRQ/VRQ		
PG0665/09	Healthy	Ovine	VRQ/VRQ		
PG0643/09	Healthy	Ovine	VRQ/VRQ		
PG0670/09	Healthy	Ovine	VRQ/VRQ		
PG0648/09	Healthy	Ovine	VRQ/VRQ		
PG1474/10	Healthy	Ovine	AHQ/AHQ	Cheviot	
PG1376/10	Healthy	Ovine	VRQ/VRQ		
PG1384/10	Healthy	Ovine	AHQ/AHQ		
PG0143/12	Healthy	Ovine	VRQ/VRQ		APHA Weybridge
PG0146/12	Healthy	Ovine	VRQ/VRQ		APHA Weybridge
PG1418/08	Healthy	Ovine	ARQ/ARQ		
PG1409/08	Healthy	Ovine	ARR/ARR		
Bovine Substrate	Healthy	Bovine			

Table 2 Scrapie infected brain homogenates

ID	TSE	Species	Genotype	Breed	Source	Brain
PG1500/02	Scrapie	Ovine	ARQ/ARQ		Preston (Barton Hall) (RL)	Cerebellum
PG1275/02	Scrapie	Ovine	VRQ/VRQ	Welsh Mountain		Cerebellum
PG0876/04	Scrapie	Ovine	AHQ/AHQ		Shewsbury (RL)	Cerebellum
PG0336/06	Scrapie	Ovine	AHQ/ARQ		Preston (Barton Hall) (RL)	Cerebellum
PG2056/03	Scrapie	Ovine	AHQ/VRQ		Truro	Cerebellum
PG1358/05	Scrapie	Ovine	AHQ/ARQ		Sutton Bonington (RL)	Cerebellum
PG0320/05	Scrapie	Ovine	AHQ/ARQ		Thirsk (RL)	Cerebellum
PG1499/02	Scrapie	Ovine	AHQ/VRQ		Preston (Barton Hall) (RL)	Cerebellum
PG0157/05	Scrapie	Ovine	AHQ/VRQ		Carmarthen (RL)	Cerebellum
PG0179/07	Scrapie	Ovine	AHQ/VRQ		APHA Weybridge	Cerebellum
PG0217/06	Scrapie	Ovine	ARH/ARH		Preston (Barton Hall) (RL)	Cerebellum
PG01443/03	Scrapie	Ovine	ARH/ARH		Newcastle (RL)	Cerebellum
PG0380/04	Scrapie	Ovine	ARH/ARH		Newcastle (RL)	Cerebellum
PG1381/04	Scrapie	Ovine	ARH/ARH		Carmarthen (RL)	Cerebellum
PG0333/06	Scrapie	Ovine	ARQ/ARH		Preston (Barton Hall) (RL)	Cerebellum
PG0781/05	Scrapie	Ovine	ARQ/ARH		Carmarthen (RL)	Cerebellum
PG1731/03	Scrapie	Ovine	ARQ/ARH		Newcastle (RL)	Cerebellum
PG0371/04	Scrapie	Ovine	ARQ/ARH		Truro	Cerebellum
B26	Scrapie	Ovine	ARQ/ARQ			Cerebellum
PG2133/03	Scrapie	Ovine	ARQ/ARQ		Truro	Cerebellum
PG1361/05	Scrapie	Ovine	ARQ/VRQ		Luddington (RL)	Cerebellum
PG0505/05	Scrapie	Ovine	ARQ/VRQ		Aberdeen (SAC)	Cerebellum
PG0376/06	Scrapie	Ovine	VRQ/ARH		Preston (Barton Hall) (RL)	Cerebellum
PG0997/05	Scrapie	Ovine	VRQ/ARH		Carmarthen (RL)	Cerebellum
PG2069/03	Scrapie	Ovine	VRQ/ARH		Newcastle (RL)	Cerebellum
PG1667/05	Scrapie	Ovine	VRQ/ARR		Aberdeen (SAC)	Cerebellum
PG0730/05	Scrapie	Ovine	VRQ/ARR		Thurso (SAC)	Cerebellum
PG1943/04	Scrapie	Ovine	VRQ/ARR		Shrewsbury (RL)	Cerebellum
PG0784/05	Scrapie	Ovine	VRQ/ARR		Aberystwyth (RL)	Cerebellum
PG0794/05	Scrapie	Ovine	VRQ/VRQ			Cerebellum
FT5026/0022	Scrapie	Ovine	VRQ/VRQ		APHA Weybridge	
PG1276/02	Scrapie	Ovine	VRQ/VRQ		Gladestry	Cerebellum
PG0169/99	Scrapie	Ovine	VRQ/VRQ			
J2916	CH1641	Ovine	ARQ/AHQ			Cerebellum
J2935	CH1641	Ovine	ARQ/AHQ			Cerebellum
J3011	CH1641	Ovine	AHQ/AHQ			Cerebellum
KB	CH1641	Ovine	AHQ/AHQ	Cheviot		Cerebellum
PG0092/06	Atypical scrapie	Ovine	ARQ/AHQ		APHA Weybridge	Cerebellum
PG0592/07	Atypical scrapie	Ovine	ARQ/ARQ		APHA Weybridge	Cerebellum

Table 3 BSE infected brain homogenates

ID	TSE	Species	Genotype	Breed	Source	Brain	Pool
PG0137/12	Classical BSE	Ovine	AFRQ/ARQ		APHA Weybridge	Cerebellum	
PG0325/10		Ovine	AHQ/AHQ		APHA Weybridge	Cerebellum	
PG0326/10		Ovine	AHQ/AHQ		APHA Weybridge	Cerebellum	
SE1762/0013		Bovine			APHA Weybridge		4
BP-1		Bovine				Brain Stem	10
SE1929/0749		Bovine			APHA Weybridge		71
SE1945/0035		Bovine			APHA Weybridge		71
SE1762/0007		Bovine			APHA Weybridge		4
SE1946/0002		Bovine			APHA Weybridge		28
FT5026/0037		Bovine			APHA Weybridge		4
SE0253/0055		Bovine			APHA Weybridge		
Brain Pool 2 (BP-2)		Bovine					
PG0091/04		Bovine		Friesian		Brain Stem	
PG0034/04		Bovine		Aberdeen Angus		Brain Stem	
PG1184/02		Bovine		Limousin		Brain Stem	
PG1257/02		Bovine		Belgian Blue		Brain Stem	
PG1412/02		Bovine		Friesian		Brain Stem	
PG0354/03		Bovine		Friesian		Brain Stem	
PG0365/03		Bovine		Friesian		Brain Stem	
PG0449/03		Bovine		Friesian		Brain Stem	
PG0487/03		Bovine		Holstein		Brain Stem	
PG0572/03		Bovine		Friesian		Brain Stem	
SE2053/0089	H-type BSE	Bovine			APHA Weybridge		
SE1868/0010	H-type BSE	Bovine			APHA Weybridge		
SE1868/0011	H-type BSE	Bovine			APHA Weybridge		
SE2053/0106	L-Type BSE	Bovine			APHA Weybridge		
SE1868/0008	L-Type BSE	Bovine			APHA Weybridge		
SE1868/0009	L-Type BSE	Bovine			APHA Weybridge		