MOLECULAR STRAIN TYPING AND ENVIRONMENTAL PERSISTENCE OF RUMINANT TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

by

Jonathan Paul Owen BSc (Hons) MSc

A thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester
2011

Department of Biology
University of Leicester
Leicester

MOLECULAR STRAIN TYPING AND ENVIRONMENTAL PERSISTENCE OF RUMINANT TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES

Jonathan Owen

ABSTRACT

A novel thermolysin digestion method for the molecular strain-typing of ruminant TSEs has been developed which resulted in the clearance of cellular prion protein (PrP^{C}) from healthy sheep or cattle brain homogenates, while digestion of scrapie or BSE infected samples resulted in the generation of the full-length disease-related isoform, PrP^{Sc}. Using antibodies against the amino terminal region of PrP it was possible to distinguish ovine scrapie from ovine BSE, permitting the potential identification of BSE infected sheep within the UK flock.

The identification of a disease-associated, endogenously-generated fragment of PrP (termed C2) in scrapie infected sheep is also described. Absent in healthy brain homogenates, the neuroanatomical distribution of both C2 fragments and thermolysin-resistant PrP^{Sc} permitted the classification of four groups within a sample of natural scrapie cases which may be representative of scrapie strain heterogeneity in the UK.

The retention of ovine scrapie and bovine BSE prion protein during incubation with six UK lowland soils in soil-packed columns over a period of 18 months was also monitored. Data was collected on the persistence, the vertical migration, and the distribution of PrP^{Sc} in soil component fractions. PrP^{Sc} bound to all six soils, with elution being dependent on soil type. The majority of PrP^{Sc} bound irreversibly, and no migration of PrP^{Sc} was observed. Differences in PrP^{Sc} persistence were dependent on soil type and prion strain.

PrP^{Sc} persistence on a single soil at defined pH, temperature, or moisture contents indicated the initial deposition of PrP^{Sc} within the column was dependent on soil pH and persistence was inversely correlated to temperature. Reduced soil moisture content resulted in increased elution and persistence of PrP^{Sc}. Data suggests that the interaction of PrP^{Sc} with soil is a complex phenomenon dependent not only upon soil type and TSE strain but also environmental factors such as soil temperature, pH, and moisture content.

ACKNOWLEDGEMENTS

I wish to thank Dr Ben Maddison and Dr Kevin Gough for their assistance and encouragement in the completion of this thesis, and Dr Alan Cann for agreeing to take on the responsibility of my supervision following the sad loss of Professor Garry Whitelam.

I would also like to thank my partner Helen Waller for her support and help during this time, and I wish to dedicate this thesis to my family, Helen's family, and the late Peter England.

I would also like to thank my friends and colleagues at both Leicester University and Nottingham University, in particular Keith Bishop, Helen Rees, Claire Baker, Maged Taema, Emeli Nilsson, Abbie Fairs and Alex Fisher.

Finally, I would like to acknowledge the financial contribution of my employers, ADAS Ltd.

PUBLICATIONS

The following papers have been published as a result of the work undertaken for this thesis:

Maddison, B.C., Owen, J.P., Bishop, K., Shaw, G., Rees, H.C., Gough, K.C. The interaction of ruminant PrP(Sc) with soils is influenced by prion source and soil type. *Environ Sci Technol.* 2010. **44**, 8503-8508.

Owen, J.P., Maddison, B.C., Whitelam, G.C., Gough, K.C. Use of thermolysin in the diagnosis of prion diseases. *Mol Biotechnol.* 2007. **35**, 161-170.

Owen, J.P., Rees, H.C., Maddison, B.C., Terry, L.A., Thorne, L., Jackman, R., Whitelam, G.C., Gough, K.C. Molecular profiling of ovine prion diseases by using thermolysin-resistant PrPSc and endogenous C2 PrP fragments. *J Virol.* 2007. **81**, 10532-10539.

CONTENTS Page

Page

List of figures

Page

List of tables

Abbreviations

°C	degrees Celsius
AP	alkaline phosphatase
BHLB	brain homogenate lysis buffer
BSE	bovine spongiform encephalopathy
CJD	Creutzfeldt-Jakob Disease
CNS	central nervous system
CWD	chronic wasting disease
DY	drowsy strain of transmissible mink encephalopathy
EDTA	Ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FFI	fatal familial insomnia
FMC	field moisture content
FSE	feline spongiform encephalopathy
g	gram
GALT	gut-associated lymphoid tissues
GPI	gycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
HRP	horseradish peroxidase
HuPrP	human prion protein
HY	hyper strain of transmissible mink encephalopathy
IC	intracerebral
kDa	kilodalton
MBM	meat and bone meal
montmorillonite	hydrated sodium calcium aluminium magnesium silicate hydroxide
MOPS	3-(N-morpholino) propane sulfonic acid
OD	optical density
PBS	phosphate buffered saline
PBST	tween supplemented phosphate buffered saline
PK	proteinase K
PMCA	protein misfolding cyclic amplification
PNPP	p-Nitrophenyl phosphate
PoPrP	porcine prion protein
Prnp	prion protein gene
Prnp $^{0/0}$ mouse	mouse lacking a functional prion gene at both alleles
PrP	prion protein
$PrP^{27\text{-}30}$	protease-resistant core of the prion protein
PrP^{C}	cellular isoform of the prion protein
PrP^{Sc}	disease isoform of the prion protein
PVDF	polyvinyl difluoride
RNA	ribonucleic acid
rPrP	recombinant PrP
sCJD	sporadic Creutzfeldt-Jakob Disease
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SHa	Syrian hamster
SOD	superoxide dismutase
TEMED	N,N,N',N'-tetramethylethylenediamine
TMB	3,3’,5,5’ tetramethylbenzidine

TME	transmissible mink encephalopathy
Tris	tris(hydroxymethyl)aminomethane
TSE	transmissible spongiform encephalopathy
U	unit of protease activity
vCJD	variant Creutzfeldt-Jakob Disease
VLA	Veterinary Laboratories Agency
xg	gravitational force

CHAPTER 1

Introduction

1.1. Overview of transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSEs) are a range of fatal, neurodegenerative diseases affecting mammals of which the only known causative agent is proposed to be the prion protein (Prusiner, 1982). TSEs are characterised by the accumulation, particularly within the central nervous system (CNS), of aggregates of the host-encoded prion protein, referred to as PrP^{C}. Unlike other infectious disease agents the causative pathogen of TSEs has been proposed to consist solely of a mis-folded version of this host derived protein, designated PrP^{Sc} (Prusiner, 1982). The accumulation of PrP^{Sc} causes neuronal cell death leading to a characteristic spongy pathology of the CNS tissue. TSEs are characterised by relatively long incubation times, with pre-clinical incubation times for Creutzfeldt-Jakob Disease in humans possibly exceeding 30 years (Manuelidis *et al.*, 2009), and the wide range of phenotypic characteristics displayed by TSE agents has led to the proposition that PrP^{Sc} itself carries strain-specific properties encoded within its conformational structure (Bessen and Marsh, 1994; Peretz *et al.*, 2002).

Examples of TSEs include rare inherited genetic disorders such as Gerstmann-Sträussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI), spontaneous TSEs such as sporadic CJD (sCJD), and TSEs which can be acquired as a result of infection with the disease agent including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, and the most recently identified human TSE, variant Creutzfeldt-Jakob disease (vCJD).

Evidence suggests that the TSE scrapie was first documented in the UK sheep flock in 1732 (McGowan, 1922), while a comparison of scrapie cases in France over a 12 year

period found no epidemiological evidence linking scrapie cases to CJD and therefore supporting the absence of evidence that transmission to humans had occurred in the following 250 or more years (Chatelain *et al.*, 1981). It has been postulated that a change in the rendering process in the late 1970s, combined with the practice at the time of using ovine meat residues (greaves) to increase protein yields in dairy and beef cattle, resulted in an initial TSE infection event within the UK cattle population arising from ovine scrapie (Nathanson *et al.*, 1997; Taylor *et al.*, 1998). Whether this was the case or whether a spontaneous occurrence of cattle TSE (akin to sCJD in humans) in conjunction with feeding practices at the time gave rise to BSE, is unknown. In either case, it is probable that the recycling of animal tissues back into the ruminant food chain resulted in disease transmission, similar to that which had been previously observed with the human TSE kuru where transmission occurred as a result of ritualistic cannibalistic practices in the Papua New Guinean Fore tribe (for review see (Lindenbaum, 2008)).

The concern that the UK population had been exposed to the BSE agent through consumption of BSE infected meat products was highlighted by Bruce and co-workers when a possible link was proposed between the occurrence of vCJD and BSE through transmission studies in mice (Bruce *et al.*, 1997) and while the vast majority of individuals who have so far died as a result of infection were found to express methionine homozygously at codon 129, the report of single vCJD occurrence in an individual heterozygous at codon 129 has raised the possibility of a second wave of vCJD deaths (Kaski *et al.*, 2009).

The direct cost of the BSE crisis has been estimated at £3.7 billion (Beck *et al.*, 2005) while the subsequent knock-on effects with regards to public trust in food and the traceability of animals within the farming industry highlight the importance of TSEs, particularly in the UK.

Although the majority of scientific opinion accepts the concept of the misfolded prion protein as the causative agent of TSE, alternative proposals have implicated 25nm virions (Manuelidis, 2007; Manuelidis *et al.*, 2007), Spiroplasma infection (Bastian, 2005; Bastian *et al.*, 2007), an autoimmune response to PrP (Axelrad, 1998), organophosphate pesticides (Purdey, 1998), and manganese exposure arising from post-industrial environmental contamination (reviewed by (Brown, 2001)). However, the generation of misfolded recombinant murine PrP capable of producing the classic neuropathology of prion disease in mice has provided further evidence in favour of the prion hypothesis (Wang *et al.*, 2010).

1.2. The pathology of TSEs

While the examination of brains from TSE cases typically show no gross changes upon necropsy, microscopic examination typically reveals microglia- and astrogliosis, and in particular neuronal degeneration and vacuolation which are the characteristic histopathological changes of TSEs (Budka, 2003). Immunohistochemical detection of PrP^{Sc} deposits in brain material is further diagnostic evidence of prion disease with the deposition of amyloid plaques composed of aggregates of PrP a notable feature of Kuru and GSS, but is less frequently observed with sporadic CJD (Hainfellner *et al.*, 1995; Hill *et al.*, 2003).

The distribution of PrP^{Sc} and infectivity are further used for the delineation of prion diseases into two distinct groupings (Budka, 2003). In the first group (containing sporadic and iatrogenic CJD, genetic prion diseases in humans, and BSE in cattle) PrP^{Sc} and infectivity is mainly associated with the central nervous system (CNS). In the second group (containing vCJD, natural and experimental scrapie, experimental ovine BSE and CWD) infectivity and PrP^{Sc} is also associated with peripheral tissues, and most notably the lymphoid system (Hilton *et al.*, 2002; Houston *et al.*, 2008; Schreuder *et al.*, 1996), although it should be noted that heterozygotic VRQ/ARR and VRQ/ARQ genotype sheep have been observed to have reduced lymphoreticular PrP^{Sc} (Jeffrey *et al.*, 2002). At the clinical stage of disease progression the majority of PrP^{Sc} and infectivity is CNS-associated in both groups, however the role of both species and disease phenotype giving rise to this differential distribution of infectivity should be considered as an important factor when considering the potential for disease transmission.

1.3. The prion protein

The prion protein is encoded by the *Prnp* gene, and produces a mature protein varying in size between 30-39kDa dependent on species and the number of repeats of an octapeptide repeat sequence in the N-terminal region. It is highly conserved in mammalian species, and in mouse the gene encodes a 254 amino acid sequence, while in sheep and cattle the genes encode 256 and 264 amino acids respectively (figure 1.1), however variations in PrP amino acid sequences both between species and between individuals within a species can greatly affect susceptibility to prion

infection. The presence of a *Prnp* gene in chicken suggests that the *Prnp* gene existed before the speciation of mammals (Gabriel *et al.*, 1992).

The signal peptide region spanning the N-terminal amino acids 1-23 directs the protein to the endoplasmic reticulum (ER) and is cleaved shortly after translation, while the C-terminal region spanning amino acids 231 to 254 in mice is also cleaved during processing prior to the addition of a gycosylphosphatidylinositol (GPI) anchor responsible for membrane attachment of the mature PrP^{C} protein (figure 1.2). N-linked glycosylation sites at amino acid residues 180 and 196 in the mature protein mean that PrP can exist in three glycoforms, namely di-, mono-, or unglycosylated states. Recent work into the role of glycosylation of PrP^{Sc} in the maintenance of strain-specific neurotropisms or infectivity is currently inconclusive. Piro and co-workers failed to identify glycosylation as a contributing factor (Piro *et al.*, 2009), whilst work by Cancellotti and colleagues and Tuzi and co-workers utilising gene-targeted transgenic mice expressing different glycosylated forms of PrP not only identified prion glycosylation as a major factor in determining susceptibility to peripheral infection, but also as a determinant factor in the presentation of clinical symptoms and neuroanatomical distribution of PrP^{Sc} (Cancellotti *et al.*, 2010; Tuzi *et al.*, 2008).

Figure 1.1. Clustal 2.1 pairwise alignment of ovine (EMBL accession number P23907) and bovine PrP (EMBL accession number P10279) amino acid sequences. The location of proposed cleavage sites resulting in generation of prion fragment C2 and C1 discussed in this work are indicated by yellow and red highlights respectively (Dron *et al.*, 2010).

Figure 1.2. The major features of the primary structure of mouse PrP. Amino acids 1-23 are cleaved during processing. Areas marked CHO are *in vivo* glycosylation sites, and GPI indicates the attachment point of the membrane anchor. The segment marked "octapeptide repeats" contains four copies of an eight residue repeat sequence capable of copper binding. Areas marked β correspond to beta sheet structures and H1-H3 correspond to helical regions. Based on (Laws *et al.*, 2001).

Of note in the primary sequence of PrP is the octapeptide repeat region near the N-terminal region of the mature protein, consisting of repeats of the amino acids PHGGGWGQ which has been implicated in copper binding and modulation (Stevens *et al.*, 2009), as well as protection of cells from oxidative stress (Cui *et al.*, 2003). Increasing numbers of octapeptide repeats have been demonstrated to result in early onset of prion disease (Moore *et al.*, 2006) and increased copper binding properties (Stevens *et al.*, 2009). In contrast, however, deletion of the octapeptide region does not confer TSE resistance although incubation time was increased and PrP^{Sc} levels were reduced approximately 30 fold (Flechsig *et al.*, 2000). Interestingly, although Fleschsig and colleagues could not detect histopathological changes to the brains of terminally ill wild-type mice (including an absence of astrogliosis typically found in murine scrapie samples), disease pathology and astrogliosis was detected in the CNS suggesting a role for the octapeptide repeat region in modulating disease presentation within the host.

The functional role of cellular PrP has not yet been fully elucidated, although since PrP^{C} is membrane bound it would suggest that direct PrP binding partners are likely to be transmembrane and secreted proteins. As mentioned, evidence has been put forward to suggest that PrP^{C} may play a role in protecting cells from oxidative stress, since cultured neurons from *Prnp*$^{0/0}$ mice lacking a functional prion gene are more susceptible to treatment with agents such as hydrogen peroxide that induce oxidative stress than wild type neurons (Brown *et al.*, 1997b). Consistent with these results is evidence that brain tissue from *Prnp*$^{0/0}$ mice exhibit biochemical changes indicative of oxidative stress such as protein carbonyls and lipid peroxidation products (Wong *et al.*,

2001b). Indeed, it has been speculated that PrP itself possesses superoxide dismutase activity (SOD) (Brown *et al.*, 1999), however other investigators have found no evidence of SOD activity, relegating the role of PrP to a regulator of protective cellular responses to oxidative stress (Jones *et al.*, 2005b). Another potential role of PrP^C has concentrated on the localisation of membrane bound PrP^C within lipid rafts, suggesting a function in transmembrane signalling. It has been hypothesised that PrP^C may become cross-linked in these rafts, inducing a neuroprotective signal transduction pathway (Westergard *et al.*, 2007)

The most widely accepted observation is that PrP^C is a copper binding protein (e.g. (Brown *et al.*, 1997a), most notably via the histidine-containing octapeptide repeats but also at a further two sites at amino acid residues 96 and 111 in mice PrP (Jones *et al.*, 2005a). Therefore the functional role of PrP^C in copper may be as a sink for copper at the cell surface, or alternatively the protein may serve as a cell surface receptor for the influx or efflux of copper. However, previously published differences noted in copper levels between wild-type and $Prnp^{0/0}$ mice brain tissue have been disputed, suggesting that the role of PrP^C in copper regulation is still unclear (for review see (Westergard et al, 2007)). Other studies have implicated a role for manganese in prion infection in both animals and humans (Mitteregger *et al.*, 2009; Thackray *et al.*, 2002; Wong *et al.*, 2001a) and indicated that the binding of manganese to recombinant PrP resulted in conformational changes such that it was capable of catalysing the aggregation of metal-free PrP (Brazier *et al.*, 2008) in addition to inducing protease resistance (Brown *et al.*, 2000).

The prion protein has also been implicated in the regulation of sleep, since loss of function in *Prnp*$^{0/0}$ resulted in variation in circadian length compared to wild-type mice, potentially implicating loss of function of the prion protein with neurodegeneration associated with FFI (Tobler *et al.*, 1996). However, in the absence of more conclusive evidence it could be suggested that while expression of the prion protein is essential for prion infection, the apparent absence of severely deleterious phenotypes in mice absent a functional prion protein suggests that the prion does not appear to be an essential protein.

1.4. Secondary structure of PrP

PrP^{C} is characterised by a relatively high content of alpha helix structure compared to PrP^{Sc}. Pan and co-workers determined the secondary structure of purified hamster PrP^{C} by Fourier-transform infrared spectroscopy to comprise 42% α-helix content with virtually no (3%) β-sheet content (Pan *et al.*, 1993). In contrast, β-sheet content of scrapie infected hamster PrP^{Sc} was found to comprise 43% and 30% as α-helix. Subsequent nuclear magnetic resonance (NMR) studies into the autonomously folding domain comprising residues 121-231 by Riek and colleagues determined that secondary structure comprises helices from residues 144 to 154, 179 to 193, and 200 to 217, while residues 128 to 131 and 161 to 164 consist of β-strands (figure 1.3). The authors speculated that the short β-strands could act as nucleation sites for β-sheet formation upon conversion of PrP^{C} to PrP^{Sc} (Riek *et al.*, 1996).

Although originally believed to be unstructured as a result of the susceptibility of the amino terminal region of PrP^{Sc} to digestion by proteinase K (PK), the N-terminal region has recently been demonstrated to undergo conformational changes upon conversion

of PrP^{C} to PrP^{Sc} as evidenced by the detection of conformationally hidden epitopes during denaturing treatment (Yam *et al.*, 2010). Furthermore, binding of copper to the amino terminal region of PrP^{C} has been observed to result in the formation of protease resistant PrP^{C} implicating a conformational change in PrP^{C} (Kuczius *et al.*, 2004). Interestingly, Kuczius demonstrated that copper binding rendered the entire PrP^{C} molecule PK resistant; this being in contrast to the PrP^{Sc} conformer which displays only C-terminal resistance to PK, suggesting that the conformation change upon copper binding was not a simple PrP^{C} to PrP^{Sc} conversion but instead a novel conformer of PrP^{C}.

The N-terminal region has been speculated to play a role in the modulation of PrP^{C} to PrP^{Sc} conversion (Cordeiro *et al.*, 2005) and be an initial contact region between PrP^{C} and PrP^{Sc} (Sekijima *et al.*, 2003). Nunziante and colleagues identified a role for the N-terminus in subcellular PrP trafficking, since deletion of the entire N-terminal region of PrP^{C} resulted in inhibition of PrP^{C} internalisation, a prolonged cellular half-life, and a delay in the transportation through the secretory pathway to the cell surface (Nunziante *et al.*, 2003). A similar delay in secretory pathway transportation was observed in PrP constructs lacking a GPI anchor; the authors also suggested a co-operative role between C-terminal membrane anchoring and glycosylation (Walmsley *et al.*, 2001).

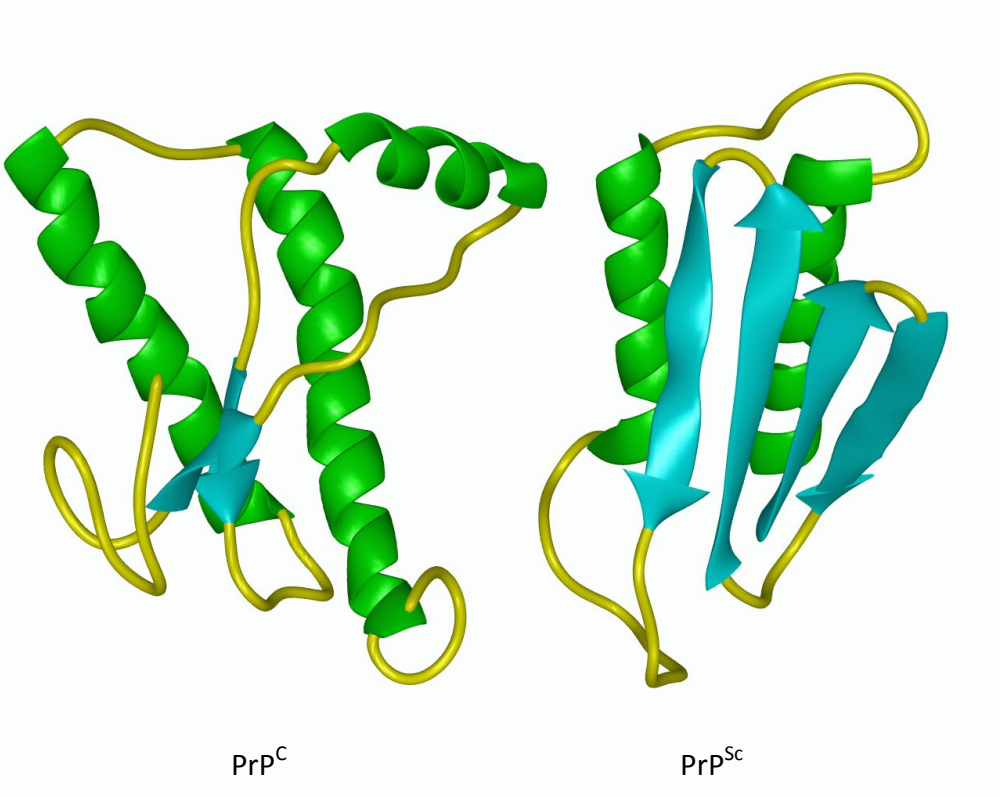


Figure 1.3. Secondary structural features of the two isoforms of the prion protein. Host encoded PrP^{C} is characterised by high α-helix content (left model) indicated in green, while the mis-folded isoform PrP^{Sc} is characterised by a much higher β-sheet content (right model) indicated in blue.

Image adapted from www.cmpharm.ecsf.edu/cohen/

1.5. Prion protein glycosylation

As noted previously, the prion protein possesses two N-linked glycosylation sites which may or may not be occupied in the mature protein and therefore PrP^{C} can exist in di-, mono-, and unglycosylated states (Endo *et al.*, 1989; Locht *et al.*, 1986). The occupation or lack thereof of these glycosylation sites gives rise to the typical triplet pattern of PrP when analysed by Western blot using PrP specific antibodies.

The effect of glycosylation on PrP^{C} function remains obscure, though many membrane targeted proteins are subjected to glycosylation (Spiro, 2002). Absence of glycosylation was found by Taraboulos and co-workers not to prevent PrP^{Sc} formation in scrapie-infected mouse neuroblastoma N2a cells (Taraboulos *et al.*, 1990), nor was an N2a cell line expressing an aglycosyl mutant form of PrP^{C} resistant to TSE infection (Korth *et al.*, 2000). Similarly, transmission of TSEs from transgenic mice lacking prion glycosylation was found to occur to wild-type mice by Tuzi and colleagues, demonstrating that glycosylation is not an essential requirement for maintenance of infectivity within a host, nor for its subsequent transmission to a host expressing normal prion glycosylation (Tuzi *et al.*, 2008).

Interestingly, Beringue and co-workers identified differing glycoform ratios in different brain regions (Beringue *et al.*, 2003), and speculated that this regional variation within the brain may play a role in the neuropathological and biological phenotype of prion strains. Furthermore, different prion strains produce different glycosylation profiles upon serial passage (Collinge *et al.*, 1996) potentially implicating particular subsets of the total PrP^{C} pool as susceptible to conversion to PrP^{Sc} in a strain dependent manner and, given the regional variation in glycoform ratios within brain regions, a potential

mechanism for the strain dependent neuropathology which is frequently observed with prion strains.

1.6. Prion conversion

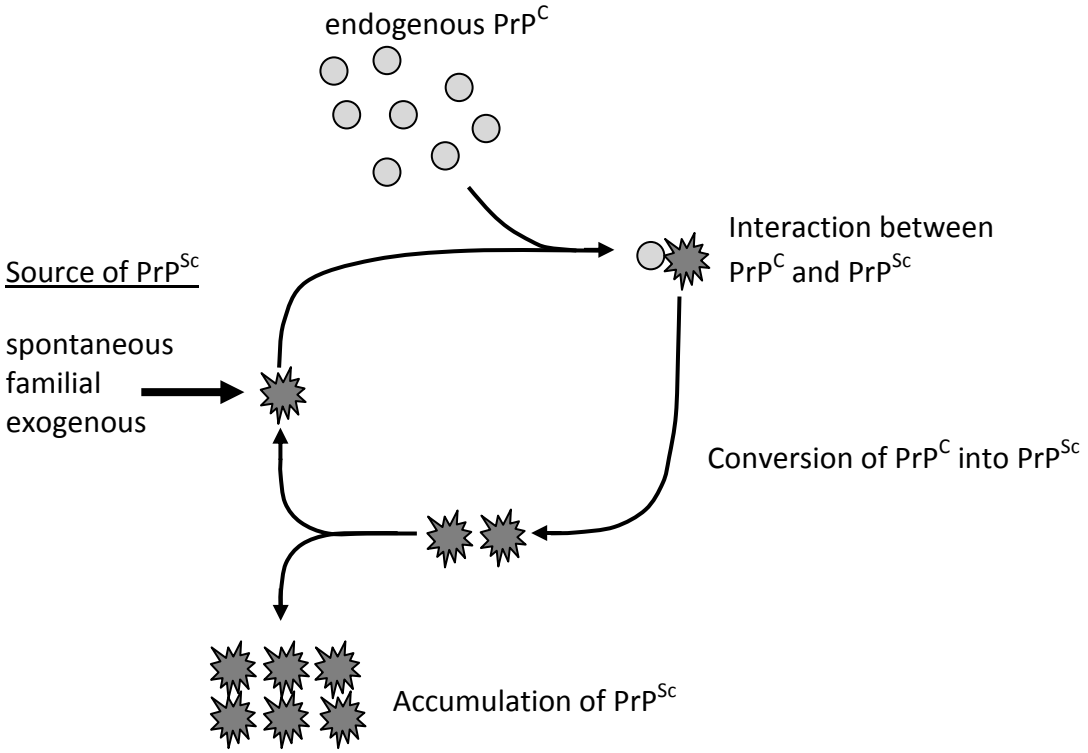
The conversion of PrP^{C} into PrP^{Sc} confers on the product a number of distinct properties capable of distinguishing the two conformers. These include detergent insolubility, a tendency to from aggregates, increased resistance to denaturation, and an increase in resistance to proteolytic digestion compared to PrP^{C} (Caughey *et al.*, 1997; Prusiner, 1998). One notable characteristic of PrP^{Sc} is a relatively high resistance to digestion with protease proteinase K compared to PrP^{C}; digestion of PrP^{Sc} with PK results in truncation in the N-terminal region and the formation of a protease-resistant core termed $PrP^{27\text{-}30}$ (McKinley *et al.*, 1983). To date the presence of PK-resistant PrP^{Sc} remains the only validated molecular marker for TSEs (Lasmezas *et al.*, 1997; Wille *et al.*, 1996).

It should be noted, however, that not all PrP^{Sc} molecules are resistant to protease digestion, as evidenced by Gambetti and co-workers who identified apparently novel CJD cases (termed protease sensitive prionopathies) characterised by the absence of proteinase K-resistant PrP^{Sc} (Gambetti *et al.*, 2008). Barron and colleagues have also demonstrated in a transgenic mouse model containing a targeted proline to leucine (101LL) mutation in the endogenous mouse PrP that levels of human GSS or scrapie strain 263K protease resistant PrP^{Sc} can be extremely low or absent while simultaneously displaying very high titres of infectivity (Barron *et al.*, 2007)

The process of conversion from PrP^{C} into PrP^{Sc} has been proposed by Prusiner to occur via an autocatalytic mechanism as a result of direct physical interaction between PrP^{C} and PrP^{Sc} (Prusiner, 1998). Other suggestions for the mechanism for PrP^{C} conversion include a heterodimer mechanism involving an intermediate conformer of PrP, termed PrP^{*} (Cohen *et al.*, 1994), possibly in conjunction with an unidentified protein (termed protein X) capable of binding to the C-terminal region of PrP and assisting in the conversion of PrP^{C} to PrP^{Sc} (Telling *et al.*, 1994). This hypothesis arose as a result of transgenic mouse studies expressing both mouse and human PrP (HuPrP); despite high levels of HuPrP expression resistance to human prion infection but not mouse prion infection was observed. This potentially indicates the presence of a species specific macromolecule preferentially binding to mouse PrP^{C} and implicated in the conversion process. However, it should be noted that protein X has never been isolated. The autocatalytic and heterodimer models have since been superseded by an alternative model proposed by Masel which incorporates a linear elongation model of PrP^{C} conversion at the ends of PrP^{Sc} fibrils with fibril breakage (and hence the generation of new fibril ends) responsible for the exponential growth in PrP^{Sc} accumulation (Masel *et al.*, 1999).

There is some evidence to suggest a role for cofactors in the conversion of PrP^{C} to PrP^{Sc}. Deleault and co-workers demonstrated that single stranded RNA was required for conversion of hamster PrP^{Sc} *in vitro*, suggesting a role for host encoded RNA in TSE pathogenesis (Deleault *et al.*, 2003). However, the same group demonstrated that these results were not applicable to a mouse model, suggesting a species-dependent requirement for ribonucleic acid (RNA) as a cofactor (Deleault *et al.*, 2010). Recent

evidence suggests that synthetic anionic phospholipid as well as RNA is required for the successful conversion of recombinant PrP (rPrP) into protease-resistant rPrP capable of causing disease in wild-type mice (Wang *et al.*, 2010).



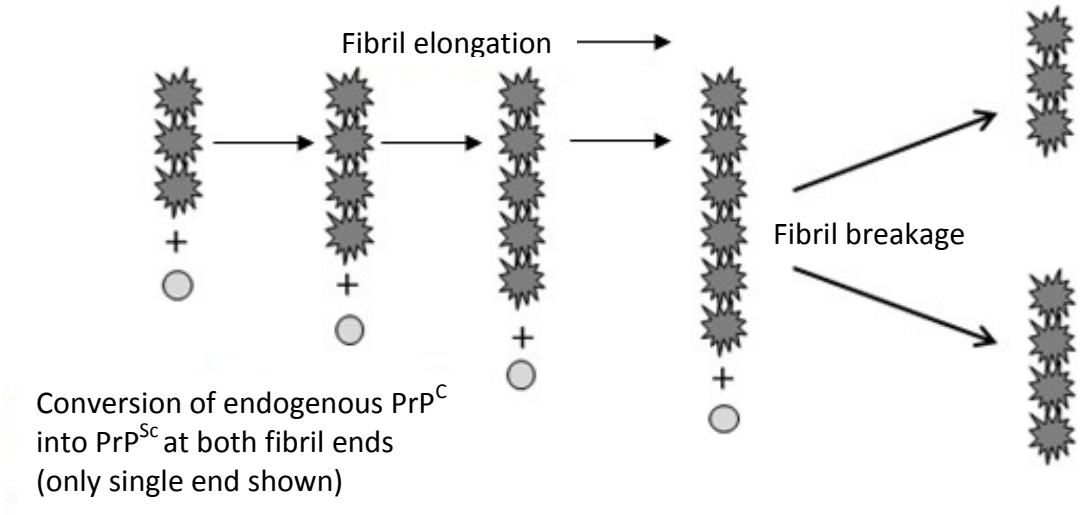


Figure 1.4. Two models of prion replication. Upper panel shows a simplistic autocatalytic replication model (Prusiner, 1998) now believed to be unrepresentative of prion replication due to the extremely high catalytic activity required of PrP^{Sc}. Lower panel shows alternative model (Masel *et al.*, 1999) with conversion of PrP^{C} at each end of PrP^{Sc} fibrils and fibril breakage which generates new fibril ends for further PrP^{C} conversion.

1.7. The species barrier

The species barrier concept arose as a result of the observation that an initial challenge of a particular species with infectious material from a second species resulted in a prolonged and variable incubation period before disease symptoms presented, and a different lesion distribution upon examination of the brain *post mortem* (Pattison and Jones, 1967). Subsequent challenges using material from the new host in the same species resulted in maintenance of the new pathology alongside shortened and predictable incubation periods. As the primary infectivity event results in *de novo* generation of PrP^{Sc} molecules with an identical amino acid sequence to the host, in subsequent passages conversion of PrP^{C} to PrP^{Sc} is favoured relative to the primary challenge. Confirmation of the species barrier was described by Scott and co-workers using mice transgenic for Syrian hamster (SHa) PrP, which showed an absence of the species barrier effect when inoculated with SHa prions. This was in contrast to non-transgenic mice, where the presence of native mouse PrP expression resulted in a species barrier upon inoculation with SHa prions (Scott *et al.*, 1989).

The species barrier is of particular importance in the UK given the BSE epidemic, since the BSE prion strain has been identified as being highly promiscuous and capable of infecting a number of mammalian species (Bruce *et al.*, 1994). Furthermore, unlike classical CJD, vCJD (and BSE) could be transmitted much more easily to wild-type mice (Hill *et al.*, 1997) suggesting not only a link between vCJD and BSE when comparing PrP deposition patterns and PrP^{Sc} digestion patterns, but also a much reduced species barrier to BSE and vCJD infection. It has also been noted that BSE in ovine and caprine hosts is transmitted more readily to mice transgenic for human PrP possessing the

Met129 allele, suggesting that not only may humans be at least as susceptible to ovine BSE as bovine BSE, but also that an intermediate passage of BSE in a ruminant host is capable of markedly increasing transmission efficiency (Padilla *et al.*, 2011).

It has been postulated that BSE arose in cattle as a result of the practice of supplementing ruminant diets with protein derived from meat and bone meal (MBM) (Smith and Bradley, 2003; Wilesmith *et al.*, 1991). These supplements were produced from the offal of cattle, sheep, pigs and chickens in order to boost yields of beef and milk, with the initial infection possibly as a result of the rendering of a spontaneous case of bovine prion disease into MBM. Indeed the identification of atypical BSE strains based on molecular profiling from different geographical regions would suggest that the spontaneous appearance of BSE is a repeated yet low frequency event (Beringue *et al.*, 2006). The presence of BSE in the UK sheep flock can also not be excluded despite an intensive search (Stack *et al.*, 2006). Although the numbers of human deaths from vCJD is relatively modest at 176 between January 1st 1990 and December 5th 2011 (source: The National Creutzfeldt-Jakob Disease Research & Surveillance Unit (NCJDRSU), Western General Hospital, Edinburgh, UK, at www.cjd.ed.ac.uk), there are considerable uncertainties in predicting the numbers of pre- or subclinical infections. These uncertainties include the potential for ovine BSE cases, the effect of human genetic differences on incubation period, the widespread UK population exposure, and secondary iatrogenic transmission mechanisms.

The absence of BSE in the UK pig population during the cattle BSE epidemic, despite exposure to the pathogen, is believed to result from a transmission barrier to infection following oral exposure (Wells *et al.*, 2003). Inclusion levels of MBM are generally

higher in commercial pig feed than ruminant rations, indicating that the UK pig population would have been exposed to relatively higher levels of BSE in feed than, for example, cattle. Notably, transmission studies to mice expressing porcine PrP (PoPrP) demonstrated complete resistance to ovine scrapie but increased susceptibility to BSE following a single passage in ARQ sheep, and susceptibility to atypical scrapie (Espinosa *et al.*, 2009). The authors also noted a shift in strain phenotype of the atypical scrapie strain analysed (SC-PS152) upon PoPrP transgenic mouse transmission in that survival times, protease-resistant PrP^{Sc} molecular profiles, and vacuolation profiles resembled sheep BSE in the same host, indicating the evolutionary potential of prions upon transmission to a novel host. Konold and co-workers were unsuccessful in oral transmission to pigs, although intracerebral inoculation resulted in the majority of animals testing negatively for PrP^{Sc} while displaying neurological symptoms of infection (Konold *et al.*, 2009). The authors concluded that there is therefore a risk that BSE may produce prion disease in pigs, but that would remain undetected by current post-mortem tests.

CWD is a growing problem in the mainland United States following the first report of a novel TSE in captive deer in the late 1970s (Williams and Young, 1980). Interspecies CWD transmission is most likely to occur in the field as a result of communal grazing areas, however both sheep and cattle appear to be poorly susceptible to mule deer CWD; of 6 sheep intracerebrally inoculated with mule deer brain suspensions harbouring CWD only 2 were infected (Hamir *et al.*, 2006), while for cattle only 3 of 13 animals inoculated tested positive (Hamir *et al.*, 2001). Human susceptibility to CWD is unknown, although epidemiological data from the region where CWD was first

identified (and hence would likely show the first incidences of human transmission) indicated that human prion disease arising from CWD was rare or non-existent (Mawhinney *et al.*, 2006).

1.8. Prion strains and strain typing

Although strains of conventional pathogens could be explained relatively easily by differences in their genomes, the apparent absence of nucleic acid involvement in preparations of material infectious for TSEs raised difficulties in explaining multiple disease phenotypes. Early evidence had suggested that preparations of infectious material exhibited considerable resistance to treatment to remove nucleic acids, suggesting that differences in disease phenotypes were either due to conformational or glycosylation differences in the protein (the protein-only hypothesis) (Prusiner, 1991) or by protein in close association with, and protecting, the nucleic acid component (the Virino hypothesis) e.g. (Dickinson and Outram, 1988).

The concept of prion strains arose as a result of the observation that multiple distinct strains of scrapie could be propagated in lines of inbred mice with distinct incubation times and patterns of neuropathology, and these characteristics could be used to classify, for example, scrapie strains (Fraser and Dickinson, 1968) and BSE (Bruce *et al.*, 2002). Although at the time Fraser and Dickenson developed this strain classification system the causative agent was poorly understood, the semi-quantitative classification of TSE strains using these methods identified a number of distinct scrapie strains, but was time consuming and required considerable skill in sample preparation and lesion density scoring.

Subsequent work identified the prion protein as the most probable causative agent (Prusiner, 1982, 1991; Safar *et al.*, 1990), and since the strain variations observed by Fraser, Bruce and other contemporary investigators (in conjunction with the identification of the prion protein as the probable causative agent of TSE infection) could not be encoded by differences in host PrP primary structure (since the use of inbred mice of the same *Prnp* genotype excluded the possibility of differences in primary PrP sequence influencing strain phenotypes), this gave rise to the suggestion that strain information was encoded by the conformation of PrP^{Sc} itself (Peretz *et al.*, 2002)

The generation of antibodies specific to PrP also permitted the use of immunohistochemical (IHC) techniques for the identification and classification of PrP deposits within the brain (Bruce *et al.*, 1989; DeArmond *et al.*, 1987; Jeffrey *et al.*, 2006b; Miller *et al.*, 1993). The use of immunohistochemical epitope mapping techniques has, for example, permitted the differentiation of BSE and scrapie in an ovine host (Jeffrey *et al.*, 2006a; Jeffrey *et al.*, 2001). More recently the use of ovinised transgenic mice has also permitted the differentiation of CH1641 and CH1641-like isolates from BSE; CH1641 and ovine BSE being notable for generating Western blot molecular profiles of unglycosylated PrP^{Sc} with similar apparent molecular mass when determined by Western blotting (Baron *et al.*, 2010; Bencsik and Baron, 2011). However IHC methods and vacuolation scoring are not suitable for analysing large numbers of samples when compared to higher-throughput methods such as Western blotting.

Besides more rapid molecular methods for strain discrimination such as Western blotting in conjunction with the limited proteoloysis of PrP^{Sc} (see section 1.9) and protein misfolding cyclic amplification (for example (Taema *et al.*, 2011)), the use of murine neuroblastoma cell lines has also permitted the rapid discrimination of prion strains in an *in vitro* assay termed the "standard scrapie cell assay" far more rapidly than traditional characterisation in mice (Mahal *et al.*, 2007).

Further evidence that prion strains were attributable to PrP^{Sc} conformation was provided by biochemical differences of PrP^{Sc} in biologically defined prion strains. Investigation into the limited proteolysis of two transmissible mink encephalopathy strains called hyper (HY) and drowsy (DY) with proteinase K indicated the possibility that PrP heterogeneity could account for strain diversity (Bessen and Marsh, 1994). Bessen and Marsh discovered differences in protease resistance and changes in the fragment size of PrP^{Sc} following digestion that implied the two strains possessed different conformations when analysed by Western blot using an antibody specific to PrP. Similarly, distinct PrP^{Sc} molecular profiles have been observed for human CJD cases when characterised by both fragment size and glycoform ratio profiles, and these differences were maintained in mice expressing murine PrP (Collinge *et al.*, 1996), demonstrating the imprinting of biochemical characteristics onto PrP from another species. Upon over-expression of human PrP in mice, however, Collinge and co-workers did identify some discrepancy when molecular profiling CJD with type-1 PrP^{Sc} from CJD cases of 129MM PrP genotype consistently converting to type-2 PrP^{Sc} upon passage to these mice expressing 129VV human PrP (Collinge *et al.*, 1996).

The maintenance of strain-specific glycoform ratio profiles upon transmission between species is worthy of note, since PrP^{C} glycosylation occurs before conversion into PrP^{Sc}. The maintenance of glycoform ratio profiles may suggest that strain specific propagation of PrP^{Sc} occurs within a particular subset of suitably glycosylated PrP^{C} from within the total PrP^{C} pool and differences in the kinetics of clearance of the un-, mono-, and diglyscosylated forms result in the final observed molecular profiles. Alternatively, aggregation of PrP^{Sc} could follow a glycosylation-dependent profile, with particular PrP^{Sc} glycoforms being incorporated into the elongating fibrils in a strain-specific ratio. Overall, it is probable that strain-specific glycosylation profiles arise as a result of a complex interaction between PrP^{Sc}, PrP^{C}, the host cell, and the cellular compartment in which formation of PrP^{Sc} occurs (Vorberg and Priola, 2002).

Evidence is emerging that prion strains actually consist of populations of "substrains" which can be selected for dependent on the host environment. Mahal and colleagues investigated the competence of 22L prions derived from brain for their ability to infect distinct R33 and PK1 cell lines, and while brain derived prions infected both cell lines, repeated propagation in PK1 cells gradually became R33 incompetent (Mahal *et al.*, 2010). When cloned R33-incompetent infectivity was returned to brain it eventually reacquired the original 22L strain characteristics. This data suggests that 22L prion populations are heterogeneous and different cellular environments can select for distinct prion variants.

1.9. Molecular strain typing of PrP^{Sc}

Various methods for the molecular differentiation of TSE strains have been described, including glycoform profiling, differential molecular mass of protease resistant PrP^{Sc}

species, resistance to chaotropic denaturation, and differential antibody binding to protease-resistant PrP^{Sc} (Baron *et al.*, 1999; Collinge *et al.*, 1996; Hill *et al.*, 1998; Pirisinu *et al.*, 2011; Stack *et al.*, 2006; Stack *et al.*, 2002). Both glycoform profiling and differential analysis of electrophoretic mobility of the prion protein rely on the generation of truncated PrP species, usually as a result of limited proteolysis of PrP^{Sc} using proteinase K (e.g. (Hill *et al.*, 1997)) combined with the subsequent detection and analysis of protein profiles by Western blotting using antibodies targeted against PrP. To date, no strain typing analyses have been performed on full length protease-resistant PrP^{Sc} in the absence of contaminating PrP^{C}, thereby excluding a region of PrP which may reveal strain related characteristics.

The ability of glycoform ratio analysis and the differential electrophoretic migration of protease-resistant PrP^{Sc} to reliably differentiate TSE strains has been questioned, with Baron and colleagues unable to differentiate ovine scrapie, bovine BSE, feline spongiform encephalopathy (FSE) and mouse passaged BSE and FSE on the basis of glycoform profiling (Baron *et al.*, 1999). They were also unable to differentiate ovine scrapie from bovine BSE on the basis of migration of the unglycosylated band when analysed by Western blot (Baron *et al.*, 1999). Similarly, Lezmi and colleagues identified ovine scrapie isolates which were incapable of being differentiated from ovine BSE on the basis of molecular mass of migration profiles (Lezmi *et al.*, 2004). Over all, multiple studies suggest that there a number of difficulties in reliably differentiating TSE strains amongst diverse host species, presumably due in part to differences in preparative and analytical methodology used among different

laboratories and the limited ability of Western blotting to reliably differentiate small differences in protein molecular mass.

1.10. Ovine scrapie

Ovine scrapie is the classical TSE disease in ruminants, and evidence from experimental transmission studies have indicated considerable biological diversity of the scrapie agents as characterised by distinct distribution of brain legions and incubation periods following sub passages in mice. Scrapie strains were first identified by Pattison and co-workers following the experimental transmission of scrapie to goats which produced dramatically different disease symptoms; clinical signs consisting of either “drowsy” or “scratching” syndromes were observed (Pattison and Millson, 1961).

While scrapie strain profiling was originally dependent on classification of incubation time and lesion profiling, more recent methods have relied on the cheaper and less time-consuming molecular methods of strain discrimination. The most common method for strain typing of TSE infected samples relies on limited proteinase K digestion of CNS homogenates to degrade PrP^{C} and the subsequent analysis of Western blot profiles of protease resistant PrP^{Sc} by detection with prion specific antibodies. Strain differentiation of the resultant profiles relies on the identification of differences in glycoform ratios (Baron *et al.*, 1999; Collinge *et al.*, 1996), PK cleavage sites resulting in changes in migration rates by Western blot (Stack *et al.*, 2006; Stack *et al.*, 2002), and/or differential antibody binding (Stack *et al.*, 2002). However, the identification of scrapie strains and their discrimination from ovine BSE is complicated by the occurrence of scrapie strains such as CH1641 which appears “BSE-like” in its

electrophoretic migration profile and antibody binding status but “ovine-like” in its glycoform ratio (Stack *et al.*, 2002), and the reduced PK resistance of atypical scrapie strain Nor98 compared to classical scrapie strains (Benestad *et al.*, 2008). Atypical scrapie cases have also been detected in elderly ARQ/ARQ sheep at a UK facility specifically established to house scrapie-free animals imported from New Zealand, suggesting that spontaneous cases of atypical scrapie occur at a low level but consistent rate (Simmons *et al.*, 2009).

Furthermore, an examination of atypical scrapie cases from Italy indicated that 69 from 70 cases were identical to the previously described Nor98 scrapie strain, however one animal (of ARQ/ARQ genotype for PrP) displayed the simultaneous appearance of both Nor98 and classical scrapie characteristics based on Western blot profiling of diseased brain regions, potentially indicating multiple infection and the maintenance of both scrapie strains within the same host (Mazza *et al.*, 2010). More recent work by Thackray and colleagues has also identified that some scrapie isolates potentially contain more than one prion strain; the strain selection apparently being dependent not only on whether the mouse host was wild-type or ovine transgenic, but also dependent on the individual mouse brain selected for transmission during prion strain typing (Thackray *et al.*, 2011).

Novel scrapie strains have also been identified in ovine genotypes previously believed to be resistant to scrapie infection (see table 1.1). Le Dur and co-workers described evidence of disease transmission of an atypical Nor98-like scrapie from ARR/ARR genotype sheep to mice over expressing ovine VRQ PrP (Le Dur *et al.*, 2005) and, while great effort has been undertaken in the UK to breed resistance to classical scrapie

through the selection of more resistant genotypes as part of the National Scrapie Plan, evidence published by Groschup and colleagues suggests scrapie infection can occur infrequently in sheep with ARR/ARR genotypes for PrP which were previously believed to confer resistance to classical scrapie and BSE (Groschup *et al.*, 2007).

Estimates for the number of reported cases of scrapie per year per million sheep of the 15 most common genotypes in the UK (2002)

Genotype	Percentage of sheep	Cases per million per year
ARR/ARR	21.3	0
ARR/AHQ	5.6	0.3
ARR/ARQ	28.0	0.4
ARR/ARH	2.1	0
AHQ/AHQ	1.9	5.0
ARQ/AHQ	6.3	8.7
AHQ/ARH	0.3	0
ARH/ARH	1.6	2.0
ARQ/ARH	1.6	5.2
ARQ/ARQ	12.2	36.9
ARR/VRQ	9.6	6.3
AHQ/VRQ	2.5	0.7
ARQ/VRQ	5.9	225.4
ARH/VRQ	0.3	405.0
VRQ/VRQ	0.9	544.5

Table 1.1. Estimates for the number of reported cases of scrapie per year per million sheep in the UK (1998-2002), and the percentage of UK sheep possessing each genotype. Reproduced from (Baylis *et al.*, 2004)

1.11. Bovine BSE

While bovine BSE was originally believed to consist of a single TSE strain, in the last 10 years novel BSE strains have been identified with differentiation based on electrophoretic migration and glycosylation profiles. The classical or "typical" BSE strain was believed to be that responsible for the UK BSE epidemic, while two further atypical BSE strains, termed H-type (Biacabe *et al.*, 2004) and L-type (Casalone *et al.*, 2004) strains, have subsequently been identified in France and Italy respectively. Classification of H-type or L-type is based upon the higher and lower molecular masses of the unglycosylated protease-resistant PrP^{Sc} band in Western blots, and in the case of L-type BSE, in conjunction with the deposition of amyloid plaques of PrP^{Sc} in the CNS; this being in contrast to the absence of amyloid plaque deposition with classical BSE.

L-type BSE has also been transmitted to a primate from an asymptomatic cattle (Comoy *et al.*, 2008), and given the similarities in biochemical and deposition patterns of L-type BSE to a subset of spontaneous CJD cases, there exists the possibility that a number of apparently spontaneous CJD cases may actually have arisen as a result of exposure to L-type BSE prions (Capobianco *et al.*, 2007). In contrast to the suggestion that vCJD originated from the classical BSE strain, phenotypic similarities between L-type BSE and a bovine passaged transmissible mink encephalopathy (TME) isolate in a transgenic ovinised mouse host has led to the proposal that TME originated from L-type BSE as a result of feeding of downer cattle on US mink farms (Baron *et al.*, 2007). Given that a number of TME outbreaks in the US have preceded the UK BSE crisis, this

would provide evidence for another example of transmission of a TSE from ruminants to non-ruminants as a result of farming practices.

In similarity to L-type BSE, H-type BSE appears to arise spontaneously within the cattle population with an estimated frequency of appearance in the UK of approximately 1 case every 2 years for H-type BSE following testing of approximately 290,000 fallen cattle aged 8 years or over in the UK between January 2005 and July 2009 (Stack *et al.*, 2009). This compares to 455 cases of classical BSE identified between 2005 and 2009 as a result of passive and active surveillance in the UK from a total cattle population of approximately 8.8 million (sources: Veterinary Laboratories Agency at http://vla.defra.gov.uk/science/sci_tse_stats_intro.htm and Defra "The Cattle Book 2008").

Transmission studies to mice transgenic for bovine PrP indicated that, in primary passage, longer incubation times were noted for H-type BSE compared to classical BSE. Furthermore, Western blot profiles demonstrated that the increase in size of unglycosylated PrP compared to typical BSE was maintained in both ovine PrP and bovine PrP transgenic mice (Beringue *et al.*, 2006). Together, these results would suggest that H-type BSE represents a second atypical strain of the BSE prion.

Serial passage of H-BSE in wild type mice was found to result in the emergence of indicators of classical BSE in some animals during the second passage (Baron et al, 2011); a similar phenomenon to that observed with L-type BSE in mice transgenic for ovine PrP whereby classical BSE phenotypes emerged (Beringue *et al.*, 2007). It should be noted, however, that in lines expressing other PrPs (including bovine PrP) the authors noted that phenotypic traits of L-type BSE were maintained. These results

underline the relationship between species-barrier crossing and prion strain diversification with the potential for strain shifting in an unpredictable manner. Whether these studies highlight a form of strain selection or alternatively a mechanism for the *de novo* generation of a novel prion strain is currently unclear.

1.12. PrP peptide markers of TSE infection

The description of a disease-associated fragment of PrP apparently generated as a result of limited N-terminal trimming of PrP^{Sc} was first identified by Chen and colleagues in brain tissue from spontaneous CJD cases, and was labelled the C2 fragment with an apparent molecular weight of approximately 20kDa (Chen *et al.*, 1995). Another PrP fragment, termed C1, and truncated more C-terminally than C2 was also identified and was associated with a constitutive cleavage process of the potentially amyloidogenic region of PrP^{C} in healthy samples. The authors speculated that the location of the cleavage site (either residues His111 or Met112 in human PrP) was a cellular mechanism to avoid the accumulation of the potentially pathogenic region corresponding to amino acids 100 to 130. In contrast, the C2 fragment has been proposed to encompass amino acids 106 to 126 and was noted to be associated only with the detergent insoluble fraction, suggesting that accumulation occurred within PrP aggregates.

C2 species have also been detected by Pan and co-workers in scrapie infected mouse brain infected with three scrapie strains (Pan *et al.*, 2005), described by the author as being 26kDa and 21kDa in size. A further faint band of 18kDa was also detected in healthy animals. As truncated forms of PrP^{Sc} have been described in multiple hosts it is therefore probable that *in vivo* generation of truncated C2 species is a potential

marker of TSE infection. Evidence has also been published suggesting generation of C2 is dependent upon which cell or tissue was supporting prion replication ((Dron *et al.*, 2010) and for clarity, the positions of C2 and C1 cleavage sites in ovine PrP as determined by Dron and co-workers have been indicated in figure 1.1) and that cleavage of C2 and a fragment of 14kDa cleaved C-terminally to the C2 cleavage region (termed CTF14) may be strain specific (Nicot and Baron, 2010).

However, until a wider range of TSE strain and host combinations have been examined for the presence of truncated PrP species and more data is obtained into the role of endogenous *in vivo* truncation of PrP^{C} and PrP^{Sc} definitive conclusions cannot be drawn.

1.13. Prion uptake and transmission

Infection of deer and sheep by TSE agents is most likely to occur through the oral route, although scarified mucosal membranes, conjunctiva and scarified skin are also suggested as alternative routes of infection (Denkers *et al.*, 2011; Hamir *et al.*, 2008; Mohan *et al.*, 2004). Uptake of prions across the mucosal membrane is believed to occur via three potential mechanisms: via M cells present in the follicle-associated epithelium of the tonsils and gut which specialise in the transport of macromolecules and particles across the epithelium (Heppner *et al.*, 2001), secondly as a result of truncation with digestive enzymes and formation of complexes with ferritin and subsequent endocytosis by a ferritin dependent pathway (Mishra *et al.*, 2004), and thirdly by dendritic cells through dendritic processes inserted in to the gut lumen for antigen capture (Rescigno *et al.*, 2001). Following uptake, PrP^{Sc} and infectivity accumulate in the gut-associated lymphoid tissues (GALT) and Peyer's patches of the

intestines (Andreoletti *et al.*, 2000; Safar *et al.*, 2008). Following transport to lymph nodes infectivity is then believed to enter the enteric nervous system, then via the parasympathetic and sympathetic efferent nerves, to the central nervous system (Andreoletti *et al.*, 2000; van Keulen *et al.*, 2008). Infectivity subsequently spreads to other tissues via the peripheral nervous system and secondary sites of prion replication appear (Jeffrey and Gonzalez, 2007; Lawson *et al.*, 2010).

Contrary to the above broad pattern of prion uptake and dissemination observed with ovine scrapie, CWD, experimental ovine BSE and vCJD, in bovine BSE there appears to little involvement of the lymphoid tissue apart from the GALT tissues of the Peyer's patches in the ileum and tonsils with infectivity largely restricted to the CNS (Espinosa *et al.*, 2007). Several groups have published data suggesting that infectivity is associated with blood in vCJD patients (Peden *et al.*, 2004), scrapie and experimental ovine BSE (Hunter *et al.*, 2002), and CWD (Mathiason *et al.*, 2010), suggesting that dissemination of infectivity is likely to more likely to occur with these four TSEs than with bovine BSE.

1.14. Prions in the environment

It is becoming increasingly clear that environmental reservoirs of CWD and scrapie exist which are capable of initiating TSE infection in susceptible species. Potential sources of these environmental reservoirs include the disposal of farm animals and the natural deposition of carcasses of wild animals. CWD prions have been shown to cause infectivity following reintroduction of mule deer into paddocks previously used to house orally infected mule deer or CWD infected carcasses, suggesting that CWD prions can persist in the environment for a period of at least 2 years (Miller *et al.*,

2004), and buried mouse adapted prions have also maintained infectivity in soil for at least 3 years (Brown and Gajdusek, 1991). Furthermore, some evidence exists to suggest that scrapie prions may have persisted in an Icelandic farm building for a period of approximately 16 years (Georgsson *et al.*, 2006). Sources of environmental contamination, aside from carcasses from fallen animals, include shedding from infected animals in faeces (Safar *et al.*, 2008), urine (Murayama *et al.*, 2007), milk (Maddison *et al.*, 2009), blood (Terry *et al.*, 2009), and saliva (Maddison *et al.*, 2010c), all of which have been demonstrated to contain scrapie PrP^{Sc}. Similarly, cervid-associated infectivity has been reported in faeces (Tamguney *et al.*, 2009), urine (Haley *et al.*, 2009b), and saliva and blood (Mathiason *et al.*, 2006). To date, no shedding of the BSE agent into the environment has been reported, suggesting that horizontal transmission of BSE in cattle may not occur (Doherr, 2003).

Cumulatively the evidence suggests that horizontal transmission of ovine scrapie and cervid CWD occurs as a result of shedding of infectivity from infected animals to form a reservoir of infectivity within the environment, and that this reservoir of infectivity can persist for long periods while remaining viable. A likely candidate for the reservoir of infectivity is soil. Grazing animals are known to ingest soil both deliberately to acquire nutrients such as calcium or sodium and incidentally (Hui, 2004). The quantities ingested by cattle may be in the order of several hundred grams per day (Mayland *et al.*, 1977), thus presenting a potential pathway for TSE infectivity uptake into new hosts.

1.15. Binding of prions to soil minerals

Given the evidence for environmental reservoirs of infectivity detailed above the binding, persistence and elution of prions from soil and soil minerals has received considerable study. However the majority of publications have studied rodent adapted prion or recombinant PrP.

Various studies have demonstrated that recombinant PrP and rodent adapted prions can adhere to soil and soil minerals and that infectivity can be maintained over extended periods (Cooke *et al.*, 2007; Jacobson *et al.*, 2010; Johnson *et al.*, 2007; Johnson *et al.*, 2006; Saunders *et al.*, 2009a; Seidel *et al.*, 2007). Seidel and co-workers established that PK-resistant PrP was recoverable from soil samples following 29 months co-incubation on soil. A similar period of persistence of infectivity was obtained by Brown and Gajdusek following the internment of TSE infected hamster brain material in garden soil for 36 months (Brown and Gajdusek, 1991).

Studies investigating the interaction between soil and PrP^{Sc} have established that binding is effectively irreversible and relatively harsh conditions, such as anionic surfactants, are required to elute PrP^{Sc} and even these are not fully effective (Cooke *et al.*, 2007; Johnson *et al.*, 2006). No elution of PrP^{Sc} was observed by Jacobson and co-workers using simulated rainwater as an eluent to elute hamster-adapted TME PrP^{Sc} bound in soil columns, suggesting that in an environmental setting extended washing of soils with rainwater alone may be insufficient to induce the migration of infectivity to soil levels below those accessible to grazing animals (Jacobson *et al.*, 2010). A recent study has investigated the potential for enzymatic treatment of CWD bound soil which, although impractical for the treatment of large areas of soil, may be

relevant as a means for the elimination of “hot spots” of infectivity (Saunders *et al.*, 2010).

Importantly, Johnson and co-workers discovered that not only did prions retain oral infectivity when bound to soil particles, but also that the oral infectivity of hamster scrapie prions was enhanced upon binding to soil, suggesting either a conformational change resulting in an increase in infectivity, or more likely the formation of soil/prion complexes were more suited for intestinal uptake compared to prion alone (Johnson *et al.*, 2007). Furthermore, truncation of PrP through the loss of an N-terminal region was observed upon elution of PrP^{Sc} from montmorillonite (hydrated sodium calcium aluminium magnesium silicate hydroxide) using a hot detergent treatment. The authors did not determine the fate of the N-terminal region, which presumably remained adhered to the soil, however binding of PK-truncated PrP to soil was not inhibited suggesting that the N-terminal region is not a necessary requirement for PrP binding to soil.

Work by Seidel and co-workers established that aqueous washes prepared from scrapie infected soil also retained infectivity (Seidel *et al.*, 2007), although most reports demonstrate an absence of recoverable PrP^{Sc} from soil washes (Jacobson *et al.*, 2010; Maddison *et al.*, 2010b). Similarly, Saunders and co-workers detected TME PrP^{Sc} in the aqueous fraction (representing some 40% of the initial dose after 60 days) following binding in buffered soil suspensions (Saunders *et al.*, 2009b). It should be noted however that binding of TME PrP to the soil fraction was also notably slower than data published by other workers who established that the interaction between soil minerals and PrP is both rapid and efficient (Jacobson *et al.*, 2010; Johnson *et al.*,

2006; Maddison *et al.*, 2010b). Overall this data would suggest that binding of PrP^{Sc} and infectivity to soil is a high affinity process and may result in infectivity remaining at or near the site of deposition, although this is subject to the caveat that matrix effects associated with environmental relevant sources of infectivity may not replicate the use of brain homogenates, rPrP or purified PrP^{Sc} typically employed experimentally.

Evidence suggests that elevated levels of clay minerals may result in an increase in the probability of CWD infection in affected areas (David Walter *et al.*, 2011), implicating soil type in the persistence of infectivity of prions in soil. Interestingly, Cooke and co-workers noted increased elution of murine PrP^{Sc} from sandy soils and low level elution from clay soils along with N-terminal truncation of PrP^{Sc} from this soil type (Cooke *et al.*, 2007).

Overall, published data indicates that soil is likely to be an important environmental reservoir of TSE infectivity and is likely to play a role in the horizontal transmission of ovine scrapie and cervid CWD. The limited mobility of PrP^{Sc} upon application to soil would further suggest that infectivity will probably remain biologically accessible within the upper soil layers, and that the persistent exposure of soil to low levels of infectivity from excretions and secretions such as urine, faeces, blood, placenta and saliva from TSE infected ruminants may represent a source of environmental infectivity for an extended period of time.

1.16. Aims

The aims of this work were two fold. Firstly to develop a novel molecular strain typing assay capable of distinguishing prion strains within ruminant hosts and secondly to investigate the factors affecting the persistence of TSE strains in soils.

In order to develop a novel strain typing assay a range of proteases were screened for proteolytic activity against PrP^{C} and PrP^{Sc}. Digestion conditions for those selected for further study would be optimised for their ability to generate peptide fragments from PrP^{Sc} which were capable of distinguishing ovine scrapie from bovine BSE/ovine BSE when analysed by Western blotting using a range of antibodies targeted to the N-terminal and core regions of PrP.

To investigate factors affecting the persistence of TSE strains in soils: a range of six UK soils would be packed into columns and maintained at a constant temperature and moisture content. A single soil from the six which demonstrated relatively high PrP^{Sc} persistence over an initial test period would also be analysed at various temperature conditions (which could typically be expected to occur in the UK during spring, summer and autumn/winter periods), soil moisture contents, and soil pH values. For each of these soils and environmental conditions both elution and persistence of PrP^{Sc} would be monitored. Furthermore, the migration of PrP^{Sc} through the column would be monitored over the time period, along with the distribution of prion into two soil fractions; namely sand and silt/clay fractions.

Overall, this work was therefore intended to investigate TSE strain identification as well as factors influencing prion persistence in the environment. These factors are of

considerable importance to the farming community; firstly, given the considerable longevity of prions in the environment and the resulting implications for disease control on affected farms, and secondly given the potential economic cost to the farming industry should ovine BSE or novel zoonotic TSEs be identified (or misidentified) in the UK flock.

CHAPTER 2

Materials and Methods

2.1. Materials

2.1.1. Handling of TSE material

All TSE work was carried out in accordance with local and national regulations regarding the handling of potentially contaminated tissue. TSE work was carried out in a Class I or Class II microbiological safety cabinet within a derogated Containment Level 3 laboratory. Decontamination and disposal of infected material was carried out in accordance with University guidelines.

2.1.2. Sourcing of CNS material

CNS material (tables 2.1 and 2.2) from healthy and TSE-infected ruminants was obtained from the Veterinary Laboratories Agency TSE-Archive (VLA, Addlestone UK). There is no evidence that bovine prion genotype affects susceptibility to BSE infection, therefore these data were not determined and are indicated as “not applicable” in tables 2.1 and 2.2 (Hunter *et al.*, 1994). Experimental material was derived following oral dosing of sheep as described by Bellworthy and co-workers (Bellworthy *et al.*, 2005), or were from sheep identified as part of the National Surveillance Program in the case of naturally-occuring scrapie cases. BSE infected cattle were natural cases, and sheep were primary passages of experimentally infected animals. Scrapie strains CH1641 and SSBP/1 were donated by N. Hunter of the Institute of Animal Health (IAH), Neuropathogenesis Unit, Edinburgh, UK. CH1641 was passaged into sheep with an AHQ/AHQ genotype by the VLA by intracerebral inoculation, following 3 previous passages in Cheviot sheep by the IAH. SSBP/1 was passaged at least 24 times through Cheviot and Cheviot cross sheep, including a final passage in VRQ/VRQ genotype

Cheviot sheep by the VLA by subcutaneous injection. Spinal cord material was obtained from the C1-C2 region.

Scrapie infected 22A mice were obtained by serial passage of experimental sheep scrapie source SSBP/1 through VM/Dk ($Sinc^{p7p7}$) mice (corresponding to PrP encoding phenylalanine at codon 108 and threonine at codon 189); (Carlson *et al.*, 1986), and 301V infected mice were obtained by passage of BSE through VM ($Sinc^{p7p7}$) mice (Bruce *et al.*, 1994). Animals were inoculated by intracerebral injection.

All TSE-affected animals were euthanized following onset of symptoms, and brain pathology was confirmed by IHC, histology, and Western blot analysis unless otherwise indicated. Natural scrapie cases and healthy animal tissue were sampled during a 12 hour autopsy and snap frozen at -80°C unless otherwise stated. Experimentally infected animals were sampled within 60 minutes of euthanasia and samples snap frozen at -80°C.

						CNS **region**			
TSE	**host**	**natural or experimental case**	**Breed**	**genotype**	**sample ID**	**M**	**CM**	**CR**	**SC**
scrapie	ovine	natural	Cambridge	ARQ/ARQ	0575/00	+			
scrapie	ovine	natural	Warborough	ARQ/ARQ	0210/03		+	+	
scrapie	ovine	natural	Charollais cross	ARQ/ARQ	0635/03		+	+	
scrapie	ovine	natural	Suffolk cross	ARQ/ARQ	0678/03		+	+	
scrapie	ovine	natural	Welsh Hill Speckled	VRQ/VRQ	1776/02	unknown			
scrapie	ovine	natural	Swaledale	VRQ/VRQ	0455/03		+	+	+
scrapie	ovine	natural	Swaledale	ARQ/VRQ	0226/03		+	+	+
scrapie	ovine	natural	Swaledale	ARQ/VRQ	0456/03		+	+	+
scrapie	ovine	natural	Finn Dorset	AHQ/AHQ	0284/97		+	+	+
scrapie	ovine	natural	Swaledale	ARQ/ARQ	0615/03		+	+	+
scrapie	ovine	natural	Welsh Hill Speckled	VRQ/VRQ	0923/03		+	+	+
scrapie	ovine	natural	Welsh Hill Speckled	VRQ/VRQ	0925/03		+	+	+
scrapie	ovine	natural	Mule	ARQ/VRQ	0836/03		+	+	+
scrapie	ovine	natural	Welsh Mountain	VRQ/VRQ	1276/02		+	+	+
scrapie	ovine	natural	Bleu De Maine	VRQ/VRQ	1563/02		+	+	+
scrapie	ovine	natural	Welsh Mountain	VRQ/VRQ	1275/02		+	+	+
BSE	ovine	experimental	Romney	ARQ/ARQ	0394/04	+			
BSE	ovine	experimental	Romney	ARQ/ARQ	0392/04		+	+	
BSE	ovine	experimental	Romney	ARQ/ARQ	1693/03		+	+	
BSE	ovine	experimental	Romney	ARQ/ARQ	0654/04		+	+	
BSE	bovine	natural cases	Multiple (pool)	N/A	VLA+B		hindbrain including cerebellum		
healthy	ovine	N/A	Suffolk	N/D	0893/01[a]	+			
healthy	ovine	N/A	Suffolk	ARQ/ARQ	0895/01[a]	+			
healthy	ovine	N/A	Suffolk	ARQ/ARQ	0877/03		+	+	
healthy	ovine	N/A	Dorset Horn	ARQ/ARQ	1035/03		+	+	
healthy	ovine	N/A	Dorset Horn	ARQ/ARQ	1037/03		+	+	
healthy	bovine	N/A	Fresian x Holstein	N/A	2977/98[b]	unknown			
healthy	bovine	N/A	Fresian	N/A	3877/98[b]	unknown			
healthy	bovine	N/A	Fresian	N/A	0431/99[b]	unknown			
scrapie	ovine	experimental	Cheviot	AHQ/AHQ	CH1641			+	
scrapie	ovine	experimental	Cheviot	VRQ/VRQ	SSBP/1			+	

[a] samples were pooled, referred to as VLA-S after pooling

[b] samples were pooled, referred to as VLA-B after pooling

N/D not determined

N/A not applicable

M medulla

CM caudal medulla

CR cerebellum

SC spinal cord

unknown – indicates brain region information for that animal was not available from the VLA

+ indicates that medulla, caudal medulla, cerebellum or spinal cord tissue was available for analysis and, for clarification, does not indicate TSE status.

Table 2.1. List of CNS material used for the development of the molecular strain profiling assay. Tissue obtained from the Veterinary Laboratory Agency (VLA) or Institute of Animal Health (IAH) consisted of a range of genotypes and breeds from healthy, natural scrapie cases and experimentally infected BSE along with experimentally defined scrapie cases CH1641 and SSBP/1. Tissue for each particular CNS region consisting of medulla (M), caudal medulla (CM), cerebellum (CR), or spinal cord (SC) is indicated with a + where material was available for analysis and, for clarification, does not signify TSE status.

						CNS region		
TSE	host	natural or experimental case	Breed	genotype	sample ID	Brainstem	cerebellum	midbrain and brainstem
BSE	bovine	natural	Fresian	N/A	0354/03	+		
BSE	bovine	natural	Fresian	N/A	0365/03	+		
BSE	bovine	natural	Fresian	N/A	0449/03	+		
BSE	bovine	natural	Holstein	N/A	0487/03	+		
BSE	bovine	natural	Fresian	N/A	0572/03	+		
BSE	bovine	natural	Fresian	N/A	0091/04	+		
BSE	bovine	natural	Aberdeen Angus x	N/A	0034/04	+		
BSE	bovine	natural	Limousin	N/A	1184/02	+		
BSE	bovine	natural	Belgium Blue x	N/A	1257/02	+		
BSE	bovine	natural	Fresian	N/A	1412/02	+		
healthy	bovine	N/A	Galloway x	N/A	0304/06	+	+	
healthy	bovine	N/A	Limousin x	N/A	0339/06	+	+	
healthy	bovine	N/A	Hereford x	N/A	0540/06	+	+	
healthy	bovine	N/A	Simmental x	N/A	0588/06	+	+	
healthy	bovine	N/A	Jersey	N/A	0670/04	+	+	
healthy	bovine	N/A	Hereford	N/A	0697/06	+	+	
healthy	bovine	N/A	Fresian x Holstein	N/A	0916/06	+	+	
healthy	bovine	N/A	Fresian	N/A	1019/06	+	+	
healthy	bovine	N/A	Charolais	N/A	1059/06	+	+	
healthy	bovine	N/A	Blonde D'Aquitaine x	N/A	1687/05	+	+	
scrapie	ovine	natural	British Friesland	VRQ/VRQ	0333/01	+	+	
scrapie	ovine	natural	Mule	VRQ/VRQ	0954/01	+	+	
scrapie	ovine	natural	Suffolk	VRQ/VRQ	1517/01	+	+	
scrapie	ovine	natural	North Country cheviot	VRQ/VRQ	1208/03	+	+	
scrapie	ovine	natural	Romney	VRQ/VRQ	1209/03	+	+	
scrapie	ovine	natural	Romney	VRQ/VRQ	1210/03	+	+	
scrapie	ovine	natural	Romney	VRQ/VRQ	1212/03	+	+	
scrapie	ovine	natural	Texel Cross	VRQ/VRQ	1207/03	+	+	
scrapie	ovine	natural	Swaledale	VRQ/VRQ	1277/09	+	+	
healthy	ovine	N/A	Cheviot	VRQ/VRQ	0949/06		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	0948/06		+	+
healthy	ovine	N/A	Suffolk	VRQ/VRQ	0765/06		+	+
healthy	ovine	N/A	Dorset	VRQ/VRQ	0618/06		+	+
healthy	ovine	N/A	Dorset	VRQ/VRQ	0503/06		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	0448/06		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	0398/06		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1700/05			+
healthy	ovine	N/A	Dorset	VRQ/VRQ	1699/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1308/05			+
healthy	ovine	N/A	Dorset	VRQ/VRQ	1301/05			+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1297/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1296/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1229/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1230/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1228/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1222/05		+	+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1225/05			+
healthy	ovine	N/A	Cheviot	VRQ/VRQ	1223/05		+	+
healthy	ovine	N/A	Dorset Horn	ARQ/ARQ	0417/03	+	+	

+ indicates that medulla, caudal medulla, cerebellum or spinal cord tissue was available for analysis and, for clarification, does not indicate TSE status.

Table 2.2. List of brain tissue type, genotype, and breed used to experimentally analyse the persistence of PrP^{Sc} in 6 UK soils. As described elsewhere, the absence of detectable PrP^{Sc} from bovine BSE cerebellum precluded its use in the analysis. All ovine scrapie samples were pooled to produce a 20% (w/v) homgenate, as were either healthy ovine, bovine BSE, or healthy bovine samples.

2.1.3. Enzymes

All proteases were obtained from Sigma-Aldrich (Poole, UK). Stock solutions were prepared at either 10x, 100x or 1000x final concentration in 1x phosphate buffered saline (PBS) and stored as aliquots at -20°C. PNGase F was obtained from New England Biolabs (Ipswich, USA).

2.1.4. Brain homogenate lysis buffer (BHLB)

Sodium deoxycholate and Nonidet type NP40 were obtained from Sigma-Aldrich. Potassium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride and potassium chloride were obtained from Fisher Scientific (Loughborough, UK). Brain homogenate lysis buffer consisted of phosphate buffered saline at pH 7.4 (containing 1.5mM potassium dihydrogen phosphate, 8mM disodium hydrogen orthophosphate, 137mM sodium chloride, and 3mM potassium chloride) prepared in distilled water, supplemented with 0.5% (w/v) sodium deoxycholate and 0.5% (v/v) Nonidet NP40. 10X phosphate brain homogenate lysis buffer at pH 7.4 (10XP BHLB) consisted of PBS containing 10X the concentrations of potassium dihydrogen phosphate and disodium hydrogen orthophosphate i.e. 15mM and 81mM respectively, again supplemented with 0.5% (w/v) sodium deoxycholate and 0.5% (v/v) Nonidet NP40.

2.1.5. Western blot analysis

Methanol, hydrochloric acid, and sodium chloride were purchased from Fisher. Trizma base, glycine, and polyoxyethylenesorbitan monolaurate (Tween-20) were obtained

from Sigma-Aldrich. Polyvinyl difluoride (PVDF) transfer membrane was obtained from Roche (Burgess Hill, UK). NuPAGE Novex protein gels, LDS sample buffer, western blotting transfer buffer, and 3-(N-morpholino) propane sulfonic acid (MOPS) gel running buffer were obtained from Invitrogen (Paisley, UK). 3MM filter paper was from Whatman International Limited (Maidstone, UK). Nonfat milk powders Marvel and SMA were purchased locally. Antibody binding and washing steps were performed in tris-buffered saline supplemented with 0.05% (v/v) Tween 20. Tris-buffered saline (1x) consisted of 25mM tris, 150mM sodium chloride, and 2mM potassium chloride, adjusted to pH 7.4 using hydrochloric acid when prepared as a 10x stock solution.

2.1.6. PrP specific antibodies

Mouse monoclonal antibody 6H4 was purchased from PRIONICS AG (Schlieren, Switzerland). Anti-PrP antibodies 8G8 and SHA31 were obtained from SPI-bio (Montigny-le-Bretonneux, France). Anti-PrP antibody P4 was originally obtained from Martin Groschup (Friedrich-Loeffler-Institute, Insel Riems, Germany) and subsequently purchased from R-Biopharm (Darmstadt, Germany). Anti-PrP antibody L42 was a gift from Martin Groschup. SAF antibodies (SAF32, SAF34, SAF84 and SAF70) were obtained from Jacques Grassi (Centro de Investigacion en Sanidad Animal, Madrid, Spain). Anti-PrP antibody AG4 was obtained from the TSE Resource Centre at the Institute for Animal Health (Edinburgh, UK). Dilutions used for ELISA and Western blotting were determined by titration.

Antibody binding motifs in relation to the primary amino acid sequence of ovine PrP are given in figure 2.1.

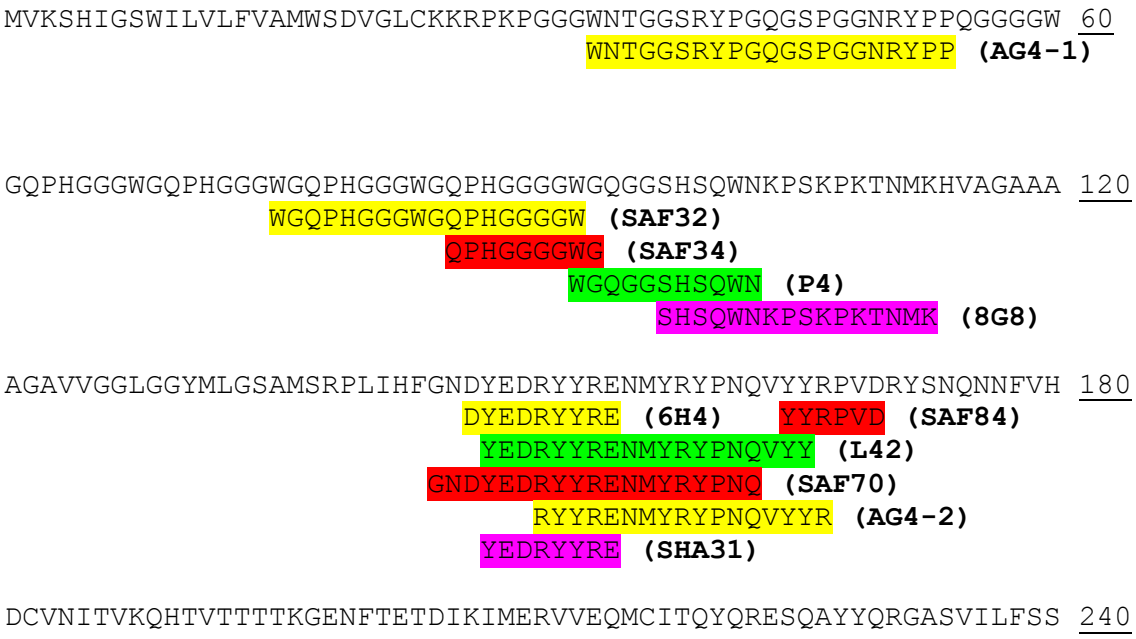


Figure 2.1. Anti-prion antibody epitope recognition sequences of antibodies AG4, SAF32, SAF34, P4, 8G8, 6H4, SAF84, L42, SAF70, and SHA31 in relation to the primary amino acid sequence of ovine prion protein (EMBL accession number P23907).

2.1.7. Anti-mouse conjugated antibodies

Goat anti-mouse horseradish peroxidase (HRP) conjugated antibody was obtained from Dako (Ely, UK). Rabbit anti-mouse alkaline phosphatase (AP) conjugated antibody was obtained from Invitrogen, along with Zymed branded rabbit anti-mouse IgG1 specific AP conjugated antibody.

2.1.8. Chemiluminescent and colorimetric substrates

BM chemiluminescence blotting substrate was obtained from Roche (Lewes, UK) and prepared according to the manufacturer's instructions. AP chemiluminescent western blotting substrate (Western Breeze) was obtained from Invitrogen. p-Nitrophenyl phosphate tablets (PNPP) for colorimetric determination were purchased from Sigma-Aldrich (Gillingham, UK) and prepared according to the manufacturer's instructions.

2.1.9. Enzyme-linked immunosorbent assay (ELISA) detection and quantification of PrP

Nunc Maxisorp 96-well flat bottomed ELISA plates were purchased from Scientific Laboratory Supplies Ltd (Nottingham, UK). Ovalbumin and Tween-20 were purchased from Sigma-Aldrich. Sodium dodecyl sulphate (SDS) was purchased from Fisher. 1X PBS was prepared as described previously. PBST consisted of 1X PBS supplemented with 0.05% Tween 20.

2.1.10. Molecular weight markers

A number of different molecular weight markers were used during the course of this work, and are indicated on each figure. Molecular weight markers included Kaleidoscope prestained protein standards and Kaledioscope prestained polypeptide

standards from Bio-Rad (Hemel Hempstead, UK), SeeBlue Plus2 protein markers and Magicmark XP protein markers from Invitrogen, and Amersham High and Low molecular weight calibration markers (GE Healthcare, Little Chalfont, UK).

2.1.11. Coomassie staining of protein gels

Gels were stained with 0.5% (w/v) Coomassie brilliant blue R250 in methanol:distilled water:glacial acetic acid 5:4:1 for 1 hour then destained with methanol:water:glacial acetic acid 7:4:1 for 3-4 hours with 4 changes of destain solution.

2.1.12. Azocoll protease activity assay

Azo dye impregnated collagen for monitoring protease activity colorimetrically and EDTA, EGTA, and N-[α-rhamnopyranosylohydroxy-phosphinyl]-Leu-Trp (phosphoramidon) for thermolysin inhibition were purchased from Sigma-Aldrich.

2.1.13. Visualisation of Western blots

Autoradiography was performed using Kodak Biomax MR film which was purchased from Sigma-Aldrich and developed using an automatic film developer using standard photographic techniques. Alternatively, chemiluminsecent western blots were visualised using an ICCD225 camera system from Photek Ltd., (St Leonards on Sea, UK).

2.2. Methods

2.2.1. Preparation of CNS homogenates

Initially, brain homogenates were prepared by a modified method of Collinge and co-workers (Collinge *et al.*, 1996). Typically 0.5 – 2g of brain material was crudely

chopped on a standard glass microscope slide using a single sided razor blade until a fine consistency had been obtained. This was transferred to a 20ml universal tube and 10 volumes of brain homogenate lysis buffer (BHLB, pH 7.4) added and the resulting suspension homogenised by passing through the body of a 2ml syringe by aspiration. This was followed by further passing through 19G, 21G, and 23G blunt-ended hypodermic needles attached to the syringe body; any connective tissue that clogged the needles was discarded. Crude cellular debris was removed by centrifugation at 500xg for 5 minutes, and the supernatant collected and stored at -20°C. Following the publication of work by Notari and co-workers (Notari *et al.*, 2004) concerning the effect of pH variation effecting proteolytic digestion of PrP^{Sc}, homogenates were prepared using the above method in 10XP BHLB at pH 7.4 containing 10X the standard phosphate concentration i.e 15mM potassium dihydrogen phosphate and 81mM disodium hydrogen phosphate.

2.2.2. Enzymatic digestion of CNS homogenate

Proteases were typically used at 50µg/ml unless indicated otherwise. In cases where activity of the supplied protease was lower than that for Proteinase K (1.6U/ml) the protease was resuspended in PBS such that the minimum activity upon addition to brain homogenate was 1.6U/ml. A complete list of proteases used to screen for proteolytic activity against PrP^{C} and PrP^{Sc} is given in table 2.3. The stated activity for each protease was based on the manufacturer's data.

Protease digestions were performed in a temperature controlled water bath (Grant Instruments Ltd., Cambridge, UK) at 37°C for all proteases with the exception of thermolysin, for which digestions were performed at both 37°C and 70°C. Reactions

were stopped by heating digestion reactions to 100°C for 5 minutes in SDS-PAGE sample loading buffer or, for thermolysin, by the addition of EDTA to a final concentration of 1mM or greater, followed by heating to 100°C for 5 minutes in SDS-PAGE sample loading buffer. Typically 25-50µl aliquots of 10% (w/v) brain homogenate were digested unless otherwise indicated.

For experiments where sequential additions of thermolysin were performed, samples were removed from the water bath after 1 hour and returned to the Class I or Class II microbiological safety cabinet. A further aliquot of thermolysin was added (typically as a 50x or 100x stock solution to avoid excessive dilution of the CNS homogenate), and the sample mixed by pipetting prior to being returned to the 70°C water bath. This procedure was repeated hourly for up to 12 hours, then digestion was stopped using EDTA and analysed by Western blot as detailed above. Where a non-metalloprotease was used for a secondary digestion following an initial thermolysin digestion, samples were treated with EDTA as described above following the initial 1 hour thermolysin digestion at 70°C. Either bromelain or protease type XIV was then added to the sample to the appropriate final concentration and incubation performed at 37°C. Samples were subsequently heated to 100°C for 5 minutes in SDS-PAGE sample loading buffer.

Deglycosylation of PrP was performed with PNGaseF using the manufacturer's protocol. Briefly, 50µl of 10% (w/v) brain homogenate was heated to 95°C for 30 minutes in the presence of 0.1 volumes of 10x PNGase F denaturing solution containing 2% w/v SDS. After cooling to room temperature 0.1 volumes of 10x G7 buffer (consisting of 250mM sodium phosphate pH 7.5) and 0.1 volumes of 10% (v/v) NP-40 were added to give final concentrations of 25mM sodium phosphate and 1%

(v/v) NP-40. 3µl (corresponding to 500 units) of PNGase F was added to the reaction mix and incubated at 37°C for 24 hours. Following deglycosylation, samples were separated by SDS-PAGE as described in section 2.2.4.

Protease	Activity (U/mg)	Concentration used
Proteinase K	32	50 µg/ml (1.6 U/ml)
Thermolysin	36	50 µg/ml (1.8 U/ml)
Pepsin A	3460	50 µg/ml (173 U/ml)
Trypsin	11470	50 µg/ml (574 U/ml)
Aminopeptidase	49	50 µg/ml (2.5U/ml)
Chymotrypsin	40	50 µg/ml (2.0 U/ml)
Kallikrein	48	50 µg/ml (2.4U/ml)
Carboxypeptidase A	50	50 µg/ml (2.5 U/ml)
Protease type XIV	4.9	320 µg/ml (1.6 U/ml)
Protease *Subtilisin carlsberg*	11.7	136 µg/ml (1.6 U/ml)
Bromelain	3.5	450 µg/ml (1.6 U/ml)
Proteinase A	20.7	77µg/ml (1.6 U/ml)
Elastase	4.9	326 µg/ml (1.6 U/ml)
Ficin	2.2	720 µg/ml (1.6 U/ml)

Table 2.3. List of proteases screened for proteolytic activity against PrP^{C} and PrP^{Sc} in 10% (w/v) ovine and bovine brain homogenates. Proteolytic activity was monitored by loss of antibody reactive signal when detected by Western blotting and chemiluminescence, as described elsewhere. Where possible, proteases were used at an identical final concentration, however those supplied at a low activity were used at the same activity as proteinase K. Activity for each protease was based on the manufacturer's supplied figures.

2.2.3. Thermolysin digestion of azo dye impregnated collagen

For monitoring the activity and inhibition of thermolysin, 1.8ml volumes of protease substrate were prepared by dissolving azo dye impregnated collagen (Azocoll) in PBS to give a final concentration of 0.5% (w/v). To these were added either 200µl PBS or 200µl of metalloprotease inhibitors consisting of EDTA, EGTA, or N-[α-rhamnopyranosylohydroxy-phosphinyl]-Leu-Trp (phosphoramidon) prepared in PBS, and incubated at room temperature for 30 minutes. Thermolysin was added to a final concentration of 50µg/ml and samples incubated at 37°C for 15 minutes with occasional shaking. Reactions were stopped on ice for 5 minutes and samples filtered through a 0.45µM acrodisc filter (Pall Corp., Portsmouth, UK). Optical densities of filtrates were recorded at 520nm on an Ultraspec 4300 Pro spectrophotometer (GE Healthcare). For analysis of the effect of inhibitors on the sequential digestion of Azocoll with thermolysin followed by protease type XIV, samples prepared as above containing inhibitor and/or thermolysin were placed on ice following the 15 minute initial incubation, protease type XIV added to a final concentration of 1.6U/ml and samples incubated for a further 15 minutes at 37°C. Reactions were subsequently placed on ice, filtered as described previously, and OD_{520} measured.

2.2.4. SDS-PAGE

A number of different SDS-PAGE gel systems were used during the course of this work including hand-cast 16% BIO-RAD mini-protean II gels, and 12% (w/v) Invitrogen NuPAGE precast gels. For BIO-RAD mini-protean II (8x10x0.07cm) gels a 16% (w/v) acrylamide: bisascrylamide (3% (w/v) bisacrylamide) gel was used, containing final concentrations of 375mM Tris-HCl pH 8.0, and 0.1% (w/v) SDS. Gels were polymerised

with 100 µl of 10% ammonium persulphate and 4 µl TEMED. The gels were overlain with a 5% (w/v) acrylamide:bisacrylamide (3% (w/v) bisacrylamide) stacking gel containing 125 mM Tris-HCl pH 6.8 and 0.1% (w/v) SDS. SDS-PAGE sample loading buffer was added to samples and heated to 100°C for 10 minutes prior to loading on to gels. SDS-PAGE sample buffer consisted of 62.5mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 0.5% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 5% (v/v) β-mercaptoethanol. Samples were electrophoresed in a buffer containing 25mM Tris base, 192mM glycine, and 0.1% (w/v) at 200V (constant voltage) for 40-60 minutes.

2.2.5. Western blotting and immunodetection

Western blotting of samples prepared for analysis by molecular strain profiling were performed using both the Invitrogen transfer system described below and the Bio-Rad semi-dry Trans-Blot system utilising Towbin buffer. Towbin buffer consisted of 25mM Tris, 192mM glycine, and 20% (v/v) methanol. Briefly, a stack consisting of 2 sheets of Whatman paper soaked in Towbin buffer was placed on the anode, the SDS-PAGE gel placed on top (following 10 minutes equilibration in Towbin buffer) and a sheet of PVDF pre-wetted in methanol followed by Towbin buffer placed on top of the gel. Two further sheets of buffer-wetted Whatman paper were placed on top of the PVDF membrane and the cathode fitted in place. Blotting was performed at 25V for 1 hour and blots rinsed briefly with TBS. Membranes were subsequently blocked for 12-16 hours at 4°C with 5% (w/v) SMA milk powder in TBS and probed with antibodies as described below.

All soil samples were analysed by electrophoresis on Invitrogen NuPAGE Novex 12% (w/v) Bis-Tris gels. Electrophoresis was performed for 1 hour at 200V constant voltage,

then the gel was removed from the gel cassette according to the manufacturer's instructions. A pre-cut sheet of PVDF (wetted with methanol then rinsed in Invitrogen NuPAGE transfer buffer) was overlaid on the gel and pre-cut sheets of Whatmann 3M filter paper overlaid on the PVDF and underneath the gel. This blotting stack was immersed in an Invitrogen NuPAGE blotting module and blotting performed for 75 minutes at 30V constant voltage. Subsequently, the PVDF membrane was rinsed briefly in 1X TBS and blocked for 12-16 hours at 4°C in 1X TBS supplemented with 5% (w/v) SMA milk powder. Anti-PrP specific antibody was diluted in TBST supplemented with 0.5% (w/v) SMA milk powder and incubated with the blots in sealed bags for 2 hours. The blots were subsequently washed 3 times (10 minutes each) in trays containing 50 ml TBST supplemented with 0.5% (w/v) SMA. Anti-mouse antibody (either alkaline phosphatase or horse-radish peroxidase conjugated) was incubated with the blots for 75 minutes, after which the blots were washed as described previously. Blots were visualised by the addition of AP substrate obtained from Invitrogen or HRP chemiluminescent substrate obtained from Boehringer Ingelheim Ltd. (Bracknell, UK) and images captured using a Photek ICCD225 camera system or Kodak Biomax MR film.

Denistometric analysis was performed using Quantiscan image analysis software from Biosoft Ltd., (Cambridge UK). Sample lanes on Westen blot autoradiographs were digitally scanned, and were used to determine signal intensity. Care was taken to ensure regions on scanned autoradiograph blots used for densitometric analysis were below the saturation level of the Biomax MR film.

2.2.6. Sourcing and location of soils from ADAS sites

Soils from 6 UK sites owned or managed by ADAS UK were obtained representing a diverse range of soil types. None of the sites had a recorded history of production animal occupation and were located in Cambridgeshire (n=3), Nottinghamshire (n=2), and Herefordshire. Precise geographic locations of the soil sampling sites are given in table 2.4.

All soils were supplied at field moisture content, and were stored at 4°C in sealed bags prior to the preparation of soil columns for the analysis of PrP^{Sc} persistence. Soil classification was based on the inorganic fraction only, and did not take into account the wide variation in organic material present in the soil, e.g. Cambs2 soil was a typical fenland peat soil from the Cambridgeshire area comprising approximately 39% (w/w) organic matter.

Soil sampling location name	OSGB Grid Reference	soil classification
Cambs1	TL43125 81979	clay loam / clay
Cambs2	TL44156 82346	clay
Cambs3	TL34211 63270	clay
Notts1	SK59061 70030	sandy loam
Notts2	SK59563 69481	loamy sand
Hereford	SO56572 48168	silt loam / silty clay loam

Table 2.4. OSGB grid references and soil classification (based on inorganic soil fraction only) of six ADAS farm sites sampled for soils used in the analysis of persistence of ovine scrapie and bovine BSE PrP^{Sc}.

2.2.7. Preparation and inoculation of soil columns

Soils were passed through a 1cm sieve on-site to remove stones and larger organic material. Prior to packing soil columns the soils were passed through a 3mm sieve to

remove finer stones and debris. Columns with a 13mm internal diameter were created by sectioning a 25ml plastic pipette plugged at the base with cotton wool to permit a 11cm soil depth with a further 2cm headspace (figure 2.1, left hand image). Soil was packed to a constant volume by the addition of pre-weighed soil in portions with tapping to settle the soil between additions, corresponding to a 14.6 cm^3 volume at bulk densities of 1.26 for Notts1, 1.36 for Notts2, 1.14 for Cambs1, 0.57 for Cambs2, 1.05 for Cambs3, and 1.42 for Hereford (g/cm^3 dry weight). No fractionation of the soils was observed during the packing procedure. Columns were seeded with 6 rye-grass seeds per column after inoculation to further mimic pasture conditions.

A B

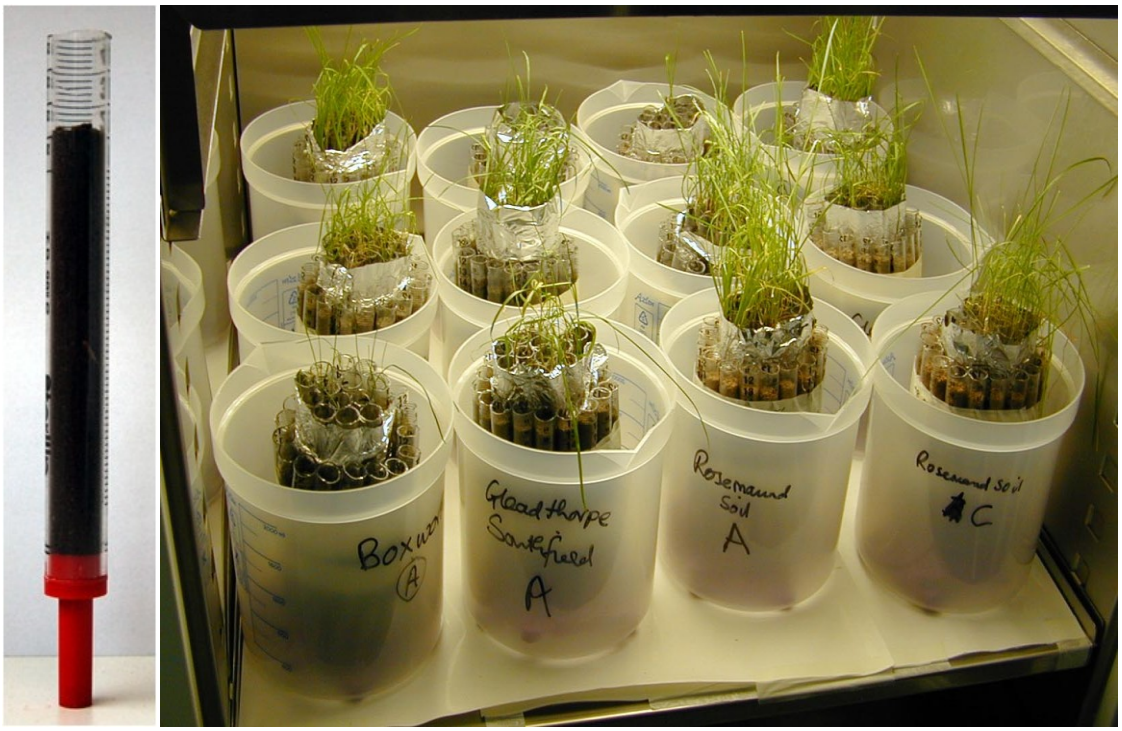


Figure 2.2. Individual soil column (panel A) and column bundles (panel B) for the analysis of PrP persistence on soil. Columns were prepared as described in section 2.2.7, inoculated with healthy ovine, scrapie ovine, healthy bovine, and BSE bovine brain homogenates and surrounded with aluminium foil. Further columns containing soil prepared in the absence of brain homogenate were used to surround the brain homogenate spiked columns and form a column bundle. Column bundles were placed in 2 litre plastic beakers and were maintained at the appropriate temperature in temperature controlled cabinets under constant illumination.

Soil columns prepared with the 6 UK soils to analyse inter-soil PrP^{Sc} persistence were incubated at 37°C prior to the addition of brain homogenate to reduce the moisture content of the soil such that addition of the spike material would return the moisture content to field moisture content. The total mass of each bundle of soil columns was determined daily and when the appropriate moisture content had been achieved, the columns were covered at the top with Parafilm and then clingfilm and stored at 4°C.

For the investigation into the persistence of PrP^{Sc} in Notts1 soil over a range of environmental conditions the requirement to reduce the soil moisture content of some columns to levels of 30% and 60% below field moisture content (FMC) limited the ability to further reduce moisture content to allow for the addition of brain homogenate. Therefore, for those columns which were to be maintained at FMC over the course of the experiment brain homogenate was added when the columns were at FMC. For those columns to be maintained at either -30% or -60% below FMC (calculated as a percentage of dry soil weight), brain homogenate was applied to columns pre-dried to these levels as described previously. Initial sampling occurred within 7 days of spiking and ELISA data adjusted at this time point to reflect the differences in moisture of the soils caused by the brain homogenate. The total mass of each column bundle was monitored for evaporative losses and subsequent samples taken when all columns had returned to pre-inoculation mass. ELISA data for soil columns maintained at -30% and -60% below field moisture content were further adjusted to equalise all readings to field moisture content in order to account for the greater soil:water ratio in these particular samples.

2.2.8. Maintenance of soil columns

Column bundles, initially consisting of 14-16 inoculated soil columns, were wrapped in aluminium foil to prevent algal growth between the soil and the column body, and surrounded with further soil-filled columns to limit temperature fluctuations. This bundle was placed in a 2 litre plastic beaker to collect any flow-through generated during column spiking or watering. Soil columns were inoculated at room temperature and incubated post-inoculation at their appropriate experimental temperatures prior to sampling. Soil columns were incubated in Snyjders temperature controlled growth cabinets (Tilburg, Netherlands) fitted with fluorescent tubes and maintained under conditions of constant illumination. For the analysis of PrP^{Sc} persistence on 6 UK soils the columns were maintained at 16-20°C for the course of the experiment. For the analysis of PrP^{Sc} persistence over a range of environmental conditions columns were maintained at 25-30°C, 8-12°C, and finally 4°C with a further incubation at -5°C for 24 hours once per month. Over the course of both experiments the total mass of each soil column bundle was determined every 7-14 days and water losses due to evaporation replaced with distilled water applied at the top of the column.

2.2.9. Adjustment of Notts1 soil pH

Soil pH was adjusted prior to column packing by the addition of ammonium sulphate (to lower soil pH) or calcium carbonate (to raise soil pH) to 1kg aliquots of sieved soil. Typically 0.385g/kg ammonium sulphate or 2.69g/kg calcium carbonate were required to adjust soil pH by 1 pH unit. Soil pH was measured 24 hours after soil pH adjustment by mixing 2g of pH adjusted soil with 10ml of distilled water for 10 minutes. Following settling of soil particles, the soil supernatant pH was determined. Soil pH values used

when preparing columns were pH 4.5, 6.2, and 7.6 for low, standard (i.e. in-the-field pH), and high pH respectively.

2.2.10. Spiking of soil columns with CNS homogenate

Homogenates from all heathy ovine animals (n=20, consisting of brain stem and cerebellum), healthy bovine animals (n=10, consisting of brain stem and cerebellum), scrapie infected ovine animals (n=9, consisting of brain stem and cerebellum), and BSE infected bovine animals (n=10, consisting of brain stem only) were pooled to provide sufficient inoculum for the addition of 10µl of 20% (w/v) CNS homogenate per 100mg soil for the 6 UK soils under analysis, and 9.7µl 20% (w/v) CNS homogenate per 100mg for Notts1 soil maintained under varying environmental conditions. No cerebellum tissue was used from BSE infected bovine animals as PrP^{Sc} was undetectable by Western blotting following proteinase K digestion and probing with anti-PrP monoclonal antibody (data not shown). CNS homogenates were stored at -80°C between preparation of the homogenates and inoculation of the soil columns.

20% (w/v) CNS homogenate prepared as described in section 2.2.1 in distilled water containing 0.5% (w/v) DOC and 0.5% (v/v) NP40 was applied to the soil surface of the soil-packed columns. For those soil types capable of absorbing the entire spike volume the 20% (w/v) brain homogenate was applied over an approximately 1 hour period. For the two soils composed of a relatively high proportion of clay minerals (Hereford and Cambs1), saturation of the upper layer occurred slowing the absorption of the spike into the column, and as a result CNS homogenate was added until saturation occurred and the remaining spike was applied 24 hours later when the initial spike had soaked into the soil. Initial sampling from columns consisting of the 6 UK soils

occurred 24 hours after the final addition of brain homogenate to each particular soil type. Subsequent sampling (from 1 month post-spiking) was synchronised. For Notts1 soil maintained under varying environmental conditions the initial sampling occurred within 7 days of the final application of brain homogenate. Subsequent samples were taken synchronously when all soil columns had returned to their original pre-spike masses.

2.2.11. Sampling and storage of CNS homogenates bound to soil

Soil columns were initially sampled within 7 days post-spiking, with further samples taken after approximately 1, 3, 6, 12, 15, and 18 months post-spiking. Prior to sampling, all soils were returned to their original field moisture content (or to 30% below or 60% below field moisture content where applicable) by the addition of water as described previously. Soil columns were cross-sectioned into 3 equal portions representing the top third, middle third, and bottom third of each column using a heated blade to score the outside of the plastic columns. Each fraction was ejected from the column section into a 9cm plastic Petri dish and homogenised using a spatula and aliquots from these three fractions were stored at -80°C. The remaining soil from the three Petri dishes was pooled and homogenised corresponding to soil from the whole of the column, and aliquots of this stored at -80°C.

2.2.12. Fractionation of soil samples into <63 µm and >63 µm fractions

1g of soil taken from the entire column depth was resuspended to a 20% (w/v) slurry in 5mM sodium hexametaphosphate and 4mM anhydrous sodium carbonate for 10 minutes on a benchtop rotator. Following centrifugation at 800xg for 10 minutes the

soil was resuspended in 5ml of 5mM calcium chloride to give a 20% (w/v) soil slurry and mixed for a further 3 hours. This slurry was poured on to a 63µm stainless steel sieve (Fisher, UK) and washed through with 45ml of deionised water. The flow through was collected in a 50ml centrifuge tube and represented the <63µm fraction. The sieve was inverted and the remaining fraction (>63µm) which had not passed through the sieve collected into a fresh 50ml centrifuge tube using 50ml of deionised water to wash the soil particles from the sieve. Both fractions were pelleted by centrifugation at 800xg for 10 minutes, and the supernatant discarded. PrP was eluted from the soil as described in section 2.2.13.

2.2.13. Extraction of soil samples for Western blotting and ELISA

PrP was eluted from soil for Western blotting using a modified protocol of Johnson and co-workers (Johnson *et al.*, 2006). 500mg soil was resuspended to a 20% (w/v) slurry in 2.5ml 5mM calcium chloride for 5 minutes on a benchtop rotator, then pelleted by centrifugation in a Beckman swing-out centrifuge at 800xg for 10 minutes. The supernatant was analysed for un-bound PrP^{Sc} or discarded where indicated. The pellet was resuspended in a further 2.5ml 5mM calcium chloride for 1 hour, rotating as before. The slurry was overlaid on a 10ml 750mM sucrose cushion prepared in 5mM calcium chloride in a 15ml polypropylene centrifuge tube. This was pelleted by centrifugation at 800xg as before to separate residual unbound PrP^{Sc} from soil-bound PrP^{Sc}. The supernatant was discarded and for Western blotting the pellet was resuspended in either 250µl of 50µg/ml proteinase K in 5mM calcium chloride for subsequent detection of the PrP^{Sc} core region, or 250µl of 100µg/ml thermolysin in 5mM calcium chloride for detection of full length or total PrP^{Sc} by ELISA. Resuspended

pellets were incubated for 1h at either 37°C for PK or 70°C for thermolysin, then an equal volume of 20% (w/v) SDS in 5mM calcium chloride was added and heated to 100°C for 10 minutes with samples subjected to 5 seconds vortexing prior to heat treatment, after 5 minutes and finally after 10 minutes. Samples were centrifuged for 10 minutes at 800xg in a Beckmann swing out centrifuge and the resultant supernatant collected in 7ml bijou tubes. The clarified supernatant was diluted 5 fold with 2.5ml ice cold methanol and precipitated by incubation at -20°C for 16 hours. Precipitated PrP^{Sc} was pelleted by centrifugation for 30 minutes at 12100xg and pellets washed with 1ml ice-cold methanol for 30 minutes by centrifugation at 12100xg. The methanol was discarded and protein pellets air dried, prior to being resuspended in 50µl 2X Invitrogen NuPAGE sample loading buffer containing 5% (v/v) β-mercaptoethanol for Western blotting or 100µl 4% (w/v) SDS in PBS for ELISA. Samples were heated to 95°C for 10 minutes and typically 20µl was loaded per well for Western blotting (equivalent to extract from 200mg of the original spiked-soil material) and 20µl was loaded per ELISA well (equivalent to 100mg of the original spiked-soil material).

2.2.14. ELISA determination of soil-eluted full-length PrP^{Sc}

Standards for ELISA consisted of the 20% (w/v) CNS homogenates used for soil spiking diluted to 10% (w/v) with 10mM calcium chloride containing 0.5% (w/v) sodium deoxycholate and 0.5% (v/v) Nonidet NP40. Aliquots of 50µl, 25µl, 10µl, 5µl, 1µl and 0µl of this 10% (w/v) CNS homogenate were made up to 50µl with the same buffer. Thermolysin was added to a final concentration of 100µg/ml and incubated at 70°C for 1 hour. EDTA was added following digestion to a final concentration of 10mM and the

samples boiled for 10 minutes in a water bath following the addition of 50µl of 20% (w/v) SDS in 5mM calcium chloride. 25µl aliquots of these digests were added to thermolysin digested, SDS eluted soil extracts from soil not previously inoculated with CNS homogenate. These standard-spiked soil extracts were precipitated with methanol as described previously and the subsequent air-dried pellet resuspended with 100µl 4% (w/v) SDS by boiling for 10 minutes and any non-solubilised material removed by centrifugation at 12100xg for 5 minutes.

Nunc maxisorp plates were coated for 16 hours at room temperature with 100µl of anti-PrP antibody SAF34 diluted 1:8000 in PBS. This solution was discarded, then wells were blocked for 1 hour with 400µl of PBST supplemented with 0.5% (w/v) ovalbumin. Following discarding of this solution, 60µl of soil-eluted PrP in 4% (w/v) SDS was diluted with 540µl PBST and the resultant 600µl added between triplicate wells and incubated for 1 hour. This solution was discarded and wells washed 5x with 400µl PBST. 100µl of SHA31 antibody diluted 1:8000 in PBST containing 0.5% (w/v) ovalbumin was added to each well and incubated for 1 hour at room temperature, then discarded, and the wells washed as before. 100µl of alkaline phosphatase conjugated rabbit anti-mouse IgG1-specific antibody diluted 1:2000 in PBST supplemented with 0.5% (w/v) ovalbumin was added and incubated for 1 hour with 5x 400µl PBST washes performed after incubation, followed by 2 further washes using 400µl distilled water. Signals proportional to the amount of bound PrP were generated by the addition of 100µl PNPP per well with absorbance readings taken after 30 minutes and 60 minutes using a Tecan Genios plate reader (Männedorf, Switzerland) at 405nm with a 620nm reference filter.

2.2.15. Bio-Rad TeSeE ELISA determination of total PrP^{Sc}

Methanol precipitated soil extracts prepared from 500mg soil as detailed in section 2.2.14 were resuspended in 100µl 4% (w/v) SDS by boiling for 10 minutes and any non-solubilised material removed by centrifugation at 12100xg for 5 minutes. 20µl aliquots were added to 180µl TeSeE sample resuspension buffer and applied to the TeSeE ELISA plate wells. Standards prepared as in section 2.2.14 were similarly resuspended in TeSeE resuspension buffer and 4% (w/v) SDS and applied to the ELISA plate. Following incubation at 37°C for 30 minutes sample wells were washed 3 times with 400µl TeSeE wash buffer and 100µl TeSeE HRP-conjugated secondary antibody added to the plate and incubated at 4°C for 30 minutes. Following 5 further 400µl washes, 100µl TMB substrate prepared according to the manufacturer's protocol was added and the ELISA plate incubated in darkness for 30 minutes. 100µl TeSeE stop solution was added and plate absorbance measured at 405nm with a 620nm reference filter.

2.2.16. Preparation of PrP^{Sc} extracts from soil for mouse bioassay

PrP^{Sc} for mouse bioassay was extracted from Notts1 soil using a modified method of Seidel and co-workers (Seidel *et al.*, 2007). Briefly, 100mg of soil was resuspended for 1 hour in 500µl PBS prior to centrifugation at 800xg for 10 minutes. The soil pellet was resuspended in 200µl of 1% (w/v) SDS in PBS and shaken vigorously for 1 hour. After centrifugation at 800xg for 10 minutes PrP^{Sc} in the supernatant was precipitated for 30 minutes at 37°C by the addition of 15µl of 4% (w/v) sodium phosphotungstic acid and 170mM magnesium chloride. PrP^{Sc} was pelleted by centrifugation at 12100xg for 30 minutes and the air-dried pellet resuspended in 20µl sterile saline prior to IC

inoculation of mice. Transgenic mice lines TgShpXI expressing ovine ARQ PrP (Kupfer *et al.*, 2007), and Tg338 expressing ovine VRQ PrP (Vilotte *et al.*, 2001) were used to determine infectivity with TSE status subsequently determined by immunohistochemistry (IHC) and vacuolation scoring of brain tissue by R.Lockey at the Veterinary Laboratories Agency (VLA). Briefly, brain sections were stained with haemotoxylin and eosin and examined by bright field light microscopy for vacuolation scoring. TSE diagnosis was based on the presence of disease-specific neuropil vacuolation and/or plaques, with all available brain regions assessed. Slides were diagnosed as positive, negative, or inconclusive; inconclusive diagnosis was on the basis of incomplete tissues or poor section levels, extensive autolysis, insufficient vacuolation or equivocal vacuolation that precluded an absolute diagnosis, or when age-associated vacuoles or autolysis made an absolute diagnosis in the presence of disease-associated vacuoles difficult. When possible, plaques were observed in the absence of possible disease specific vacuolation. Legion severity was semi-quantified by assigning a score of 0-5 for 9 specific neuroanatomical gray matter areas (G1-G9) and 0-3 for 3 specific white matter areas (W1-W3). Gray matter was scored according to the description of Beck and co-workers (Beck *et al.*, 2010). Areas scored were G1, dorsal medulla nuclei; G2, cerebellar cortex of the folia including the granular layer, adjacent to the fourth ventricle; G3, cortex of the superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septal nuclei of the paraterminal body; G8, cerebral cortex (at the level of G4 and G5); and G9, cerebral cortex (at the level of G7). White matter areas scored were W1, cerebellar white matter; W2, white matter of the mescencephalic tegmentum and W3, cerebral peduncles.

IHC was performed by labelling samples with anti-PrP rabbit polyclonal antibody Rb486 which recognises amino acids 221-233 of the bovine prion protein. All available brain tissues were assessed and a positive, negative, or inconclusive diagnosis made.

2.2.17. Preparation of PrP^{Sc} extracts from soil for protein misfolding cyclic amplification (PMCA)

For PMCA reactions, 100mg soil from each spiked soil was resuspended for 1 hour in 500µl PBS prior to centrifugation at 800xg for 10 minutes. The soil pellet was resuspended in 200µl of 1% (w/v) SDS in PBS and shaken vigorously for 1 hour. After centrifugation at 800xg for 10 minutes, 10ul (equivalent to 5mg soil) of supernatant was added to 90µl of 10% (w/v) brain homogenate (either VRQ/VRQ genotype ovine or bovine) prepared in PBS containing 150mM sodium chloride, 0.1% (v/v) Triton X-100, 4mM EDTA and 20 Mini complete protease inhibitor tablets (Roche) per litre. Amplification (performed by C. Baker (ADAS UK)) was performed in a sonicating water bath at 37°C using a Masonix S-4000 sonicator at 80% power for 40 seconds pulse time with 29 minutes 20 seconds between pulses. After every 24 hour period 10µl of sample was aliquoted into 90µl of fresh 10% (w/v) brain homogenate and returned to the sonicating water bath, and samples were amplified for 7 days in total prior to PK digestion and Western blot analysis.

CHAPTER 3

Development of a Western blot assay for the molecular profiling of ruminant TSEs

3.1. Introduction

The chapter describes the development of a novel strain typing assay capable of differentiating two TSE strains in the same host species. A number of proteases were screened for their capability to digest PrP^{C} and PrP^{Sc}, with those showing potential as the basis of a strain typing assay examined further. Initially the assay was optimised using ovine scrapie and bovine BSE samples, and subsequently the more challenging task of differentiating ovine scrapie from ovine BSE was undertaken with re-optimisation of the assay with these samples performed as necessary.

3.2.1. Screening of a range of proteases to establish susceptibility of PrP^{C} to digestion

The aim of this work was to screen a number of proteases alongside the archetypal protease for PrP profiling, namely proteinase K, for their capacity to digest PrP^{C} and PrP^{Sc} with subsequent detection by Western blot. Suitable candidates for further analysis would be selected and digestion conditions optimised to enhance the production of strain-specific differences.

The ability to digest PrP^{C} was determined to be a pre-requisite for the creation of a strain-typing assay, since residual PrP^{C} peptides would potentially complicate Western blot banding patterns when comparing TSE strains, and species-specific differences in the susceptibility of PrP^{C} to proteolytic digestion have previously been reported (Buschmann *et al.*, 1998). An assay incapable of readily distinguishing between healthy and infected materials would be unlikely to represent an improvement on current methods.

An initial screen was performed on 14 out of 15 proteases for their ability to digest PrP^C present in bovine brain homogenate, with those that resulted in digestion of PrP^C subsequently analysed for their ability to digest PrP^{Sc} both in BSE infected bovine and scrapie infected ovine brain homogenates (see table 3.1). Included within these 15 proteases was proteinase K (PK), classically used as the archetypal protease for clearance of PrP^C and the generation of protease resistant PrP^{Sc}. As described previously within this work (section 2.2.2), where possible a concentration of protease comparable to that of PK was used, however, in certain cases the activity of protease was below that described for PK, and in these cases the concentration of protease used was such that their activity was comparable to that of PK at 50µg/ml i.e. 1.6U/ml.

10% (w/v) CNS homogenate from a pool of brain stem material from 3 healthy cattle consisting of 2 Fresian and 1 Fresian x Holstein, (animals 3877/98, 0431/99, and 2977/98), was prepared in BHLB at pH 7.4 as described previously (section 2.2.1) and digested at 37°C for 1 hour with the appropriate protease with the persistence of PrP^C monitored by Western blot using an antibody specific to the core region of PrP, namely 6H4 (figure 3.1).

Of the 14 proteases tested healthy brain homogenate samples digested with pepsin A, proteinase A, aminopeptidase, kallikrein, and carboxypeptidase A appeared to have little effect on PrP^C immunoreactivity as judged by the intense Western blot signals. Although comparisons of signal intensities with the poorly defined control lanes of undigested brain homogenate was difficult, elastase and trypsin appeared to exhibit partial digestion of PrP^C, while the remaining proteases resulted in low levels of PrP^C remaining as judged by comparing to non-digested control sample. PK has traditionally

been used in the 10-50 µg/ml concentration range to clear PrP^C, so these results were in agreement with previously published findings e.g. (Collinge *et al.*, 1996).

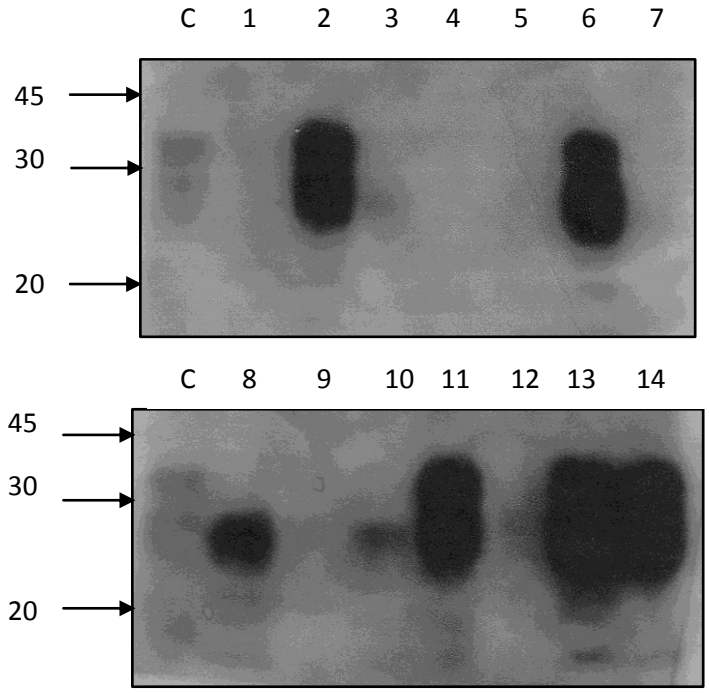


Figure 3.1. Screening 14 proteases for their capability to digest bovine PrP^C. 10% (w/v) brain homogenates prepared from a pool of healthy cattle (VLA-B) in BHLB at pH 7.4 were digested for 1 hour at 37°C and loss of immunodetectable PrP^C monitored by Western blot. 3.3µl of 2% (w/v) undigested bovine brain homogenate (corresponding to 66µg brain material) were loaded in lane C in both blots and 3.3µl of 10% (w/v) protease digested bovine brain homogenate (corresponding to 330µg brain material) were loaded in lanes 2-14. Proteases screened in panel A were: proteinase K (lane 1), pepsin A (lane 2), thermolysin (lane 3), protease type XIV (lane 4), protease *Subtilisin carlsberg* (lane 5), proteinase A (lane 6), bromelain (lane 7), elastase (lane 8), ficin (lane 9), trypsin (lane 10), aminopeptidase (lane 11), chymotrypsin (lane 12), kallikrein (lane 13), and carboxypeptidase (lane 14). Samples were separated on 16% (w/v) SDS-PAGE gels, and PrP^C detected using anti-PrP antibody 6H4 diluted 1:100,000, anti-mouse immunoglobulin-HRP conjugated antibody, and visualised using BM chemiluminescence blotting substrate. Protease concentrations used are shown in table 2.3.

3.2.2. Screening of proteases for their capability to digest ovine scrapie and bovine BSE PrP^{Sc}

Digestion of 10% (w/v) CNS homogenates from scrapie infected sheep (medulla region from a natural case of scrapie in a Cambridge sheep of genotype ARQ/ARQ identified as animal number 0575/00) and BSE infected cattle (VLA+B, consisting of hind brain including cerebellum from a number of natural BSE cases from multiple breeds and supplied by the VLA as a standardised 100% (w/v) homogenate pool) were analysed using 11 of the 15 proteases selected and indicated a number of potential candidates for further analysis (figure 3.2). Digestions of ovine scrapie and ovine BSE homogenates were not performed for some of those proteases which were incapable of clearance of PrP^{C} from healthy bovine brain homogenate, namely pepsin A, aminopeptidase, kallikrein, and carboxypeptidase. Of the candidates selected for further study the most notable was thermolysin, digestion with which resulted in the clearance of PrP^{C} but appeared not to result in the truncation of PrP^{Sc} observed with PK. A summary of this data is given in table 3.1.

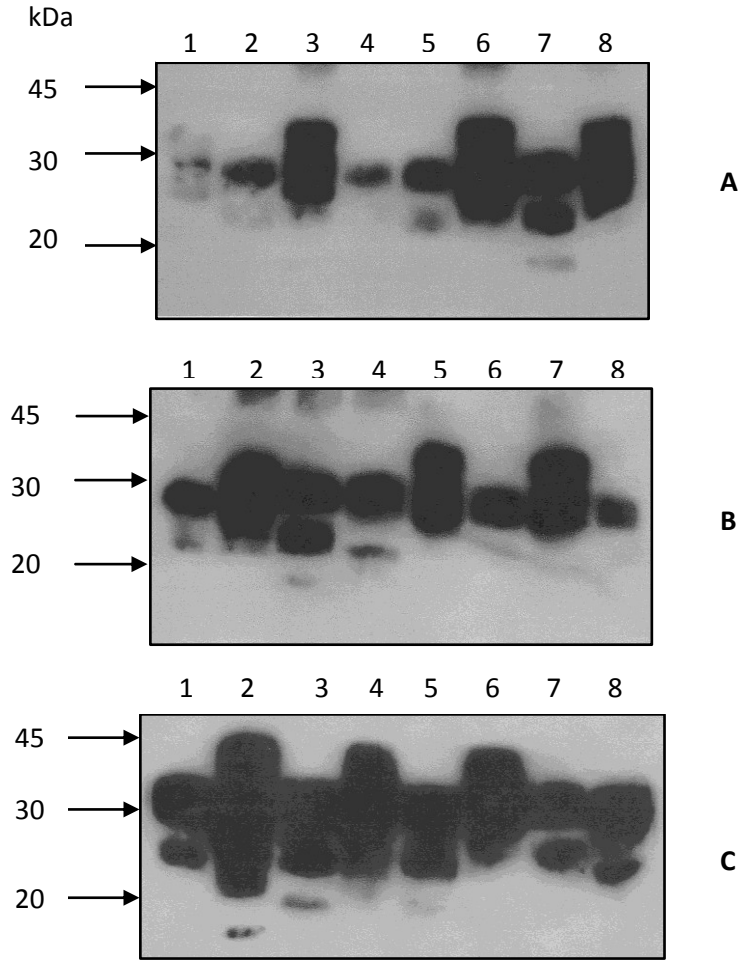


Figure 3.2. Screening of 11 proteases for their capacity to digest ovine scrapie and bovine BSE PrP^{Sc}. Brain homogenates (10% (w/v)) from scrapie infected sheep (0575/00) and BSE infected cattle (VLA+B pool) prepared in BHLB at pH 7.4 were digested for 1 hour at 37°C and loss of immunodetectable signal monitored by Western blot. For ovine samples (panel A lanes 1-8 and panel B lanes 1-4), samples were undigested (panel A lane 1) or digested with proteinase K (panel A lane 2), thermolysin (lane 3), protease type XIV (lane 4), protease *Subtilisin carlsberg* (lane 5), proteinase A (lane 6), bromelain (lane 7), elastase (lane 8), ficin (panel B lane 1), trypsin (lane 2), chymotrypsin (lane 3), or chymopapain (lane 4). For bovine samples (panel B lanes 5-8 and panel C lanes 1-8), samples were undigested (panel B lane 5) or digested with proteinase K (panel B lane 6), thermolysin (lane 7), protease type XIV (lane 8), protease *Subtilisin carlsberg* (panel C lane 1), proteinase A (lane 2), bromelain (lane 3), elastase (lane 4), ficin (lane 5), trypsin (lane 6), chymotrypsin (lane 7), or chymopapain (lane 8). Undigested samples consisted of 6.6µl of 2% (w/v) brain homogenate (corresponding to 132µg brain material) and digested samples of 6.6µl of 10% (w/v) bovine brain homogenate (corresponding to 660µg of brain material). Samples were separated on 16% SDS-PAGE gels and PrP detected using anti-PrP antibody 6H4 diluted 1:100,000, and anti-mouse immunoglobulin-HRP conjugated antibody. Signals were visualised using BM chemiluminescence blotting substrate.

Protease	Healthy bovine brain homogenate	BSE infected bovine brain homogenate	Scrapie infected ovine brain homogenate
Proteinase K	+	+	+
Pepsin A	-	N/D	N/D
Thermolysin	+	-	-
Protease type XIV	+	+	+
Protease *Subtilisin carlsberg*	+	+	+
Proteinase A	-	-	-
Bromelain	+	+	+
Elastase	+/-	-	-
Ficin	+	+	+
Trypsin	+/-	-	-
Aminopeptidase	-	N/D	N/D
Chymotrypsin	+	+	+
Kallikrein	-	N/D	N/D
Carboxypeptidase	-	N/D	N/D
Chymopapain	N/D	+	+

Table 3.1. Screening of 15 proteases for their potential for digestion of PrP^{C}, bovine BSE PrP^{Sc}, and ovine scrapie PrP^{Sc}. Ovine and bovine brain homogenates (10% (w/v)) were digested for 1 hour at 37°C and subsequently analysed by Western blot as described in figure 3.2. Table indicates scoring based on whether digestion occurred resulting in truncation of full length PrP^{Sc} to $PrP^{27\text{-}30}$ or clearance of PrP^{C} from healthy samples (+), or no apparent truncation or PrP^{C} clearance was observed (-). Intermediate digestion of PrP^{C} where incomplete clearance of PrP^{C} was observed are indicated as +/-. N/D = not determined.

3.2.3. Selection of proteases

From the initial screen of proteases for proteolytic activity against PrP^{C} and PrP^{Sc}, three were selected for further study; namely thermolysin, bromelain, and protease type XIV. As previously mentioned, thermolysin was noteworthy since digestion of healthy bovine homogenate indicated complete clearance of PrP^{C} but no apparent effect on the apparent molecular weight of PrP^{Sc} from either ovine scrapie or bovine BSE PrP^{Sc} when analysed by Western blot. It was speculated that the apparent absence of proteolysis of PrP^{Sc} compared to PrP^{C} when using thermolysin would also permit the investigation of two-step protease digestions if necessary; initial clearance of PrP^{C} using thermolysin (inhibited after digestion by the addition of EDTA) could be followed by use of other, non-metalloproteases, to produce either strain-specific peptides or differentially digested PrP^{Sc} dependent on strain; both of which offer the potential for detection by immunological methods.

Furthermore, proteinase K digestion results in the loss of the N-terminal region and the production of the core region of PrP, namely PrP^{27-30}, and therefore antibody detection of PK-digested PrP^{Sc} is reliant on the presence of epitopes located within the core region. The generation of full-length PrP^{Sc} in the absence of contaminating PrP^{C} would potentially permit not only an increase in sensitivity of PrP detection by using a combination of N-terminal specific and core-specific antibodies, but would also permit the potential development of strain typing assays using antibodies targeted against the N-terminal region since this region is known to be less structured than the hydrophobic core and therefore accessibility to digestion sites located within this region could be strain dependent.

Bromelain and protease type XIV were also selected for further analysis as they appeared to demonstrate both clearance of PrP^{C} and the truncation of both ovine scrapie and bovine BSE PrP^{Sc} resulting in protein profiles of similar apparent molecular mass to proteinase K digested samples. This offered the potential for optimisation of digestion conditions for either the generation of strain-specific peptide fragments or strain-specific differential susceptibility to digestion.

3.2.4. The use of the protease bromelain as the basis for a strain typing assay

Digestion of ovine scrapie, ovine BSE, and bovine BSE 10% (w/v) brain homogenates was performed with 1.6U/ml (77µg/ml) bromelain for 1 hour at 37°C following an initial digestion at 70°C with 50µg/ml thermolysin to remove PrP^{C}. Western blotting (figure 3.3) of the resulting samples and detection with antibodies targeted to the protease-resistant core of PrP (SAF84 or 6H4) indicated that the unglycosylated PrP band from bovine BSE (VLA+B pool) and ovine BSE (animal 1693/03) migrated with a lower apparent molecular weight than that from ovine scrapie (animal 0575/00). The difference in apparent molecular weight was small (in the order of 0.5-1 kDa), hence the identification of differences between the strains proved difficult. Similar Western blotting migration profiles were obtained using 50µg/ml PK, suggesting that this result was similar to published strain typing methods that employ PK; for example, that previously identified by Hill and co-workers (Hill *et al.*, 1998). Therefore while bromelain was capable of distinguishing ovine scrapie from ovine BSE and bovine BSE further development of this assay was not carried out due to similarity with other described assays and the inherent difficulty associated with resolving very small weight differences in Western blotted prion protein.

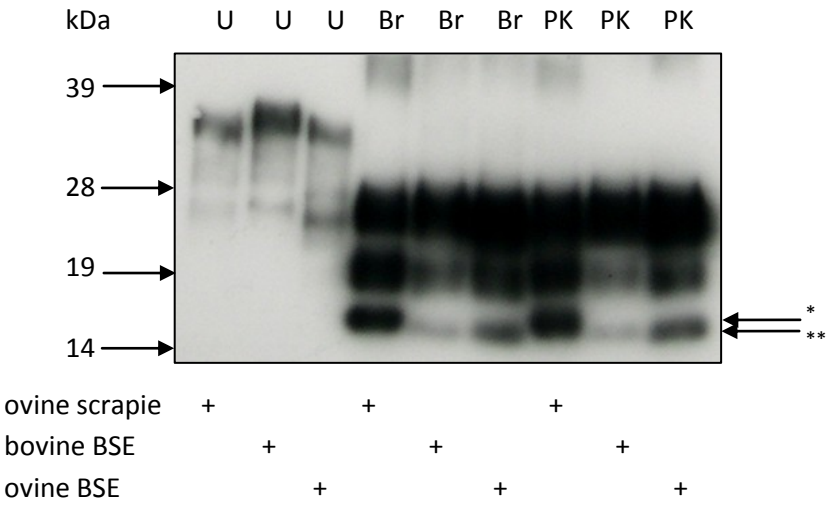


Figure 3.3. Digestion of ovine scrapie (animal 0575/00), bovine BSE (VLA+B pool) and ovine BSE (animal 1693/03) brain homogenates with bromelain or PK. Brain homogenates (10% (w/v)) from ovine scrapie, bovine BSE and ovine BSE infected animals (as indicated) were digested with 50µg/ml thermolysin for 1 hour at 70°C to remove PrP^C prior to digestion with 77µg/ml bromelain (Br) or 50µg/ml PK (PK) for 1 hour at 37°C. Samples of 6.6µl 10% (w/v) digests were loaded per lane alongside 3.3µl 2% (w/v) undigested samples (U) on 16% SDS PAGE gels. PrP was detected with anti-PrP antibody SAF-84 diluted 1:200 and anti-mouse immunoglobulin-HRP conjugated antibody. Signals were visualised using BM chemiluminescence blotting substrate. Location of ovine scrapie (*), and bovine BSE and ovine BSE (**) unglycosylated fragment locations are indicated.

3.2.5. The use of protease type XIV as the basis for a strain typing assay

Initial experiments were carried out to investigate the potential of protease type XIV to differentiate ovine scrapie from bovine BSE. Brain homogenates (10% (w/v)) from ovine scrapie animal 0575/00 and bovine BSE VLA+B pool prepared in BHLB at pH 7.4 were digested with 1.6U/ml (320µg/ml) protease type XIV for 1 hour at 37°C and analysed by Western blot using antibody 6H4 targeted against the core region of PrP.

Results indicated the presence of a doublet of approximately 18kDa and 17kDa in bovine BSE samples and a single band of approximately 18kDa apparent molecular weight for ovine scrapie (figure 3.4). Repeating this analysis using ovine scrapie 0575/00 and ovine BSE 1693/03 brain homogenates did not, however, result in the production of a corresponding doublet from the ovine BSE sample. A single band of approximately 18kDa was observed for both ovine scrapie and ovine BSE indicating that this combination of proteases and antibody was incapable of differentiating ovine BSE from ovine scrapie (data not shown).

Re-optimisation of the digestion was performed which included varying the specific pH at which the digestion was carried out (at pH 6.5 and at pH 7.5) using brain homogenates prepared in BHLB containing 10x standard phosphate levels and adjusted to the appropriate pH, and increasing the protease type XIV concentration from 1.6U/ml to 16U/ml (corresponding to an increase from 320ug/ml to 3.2mg/ml).

Digestion of ovine scrapie 0575/00 and ovine BSE 1693/03 10% (w/v) brain homogenates at the new protease concentration of 16U/ml for 1 hour at 37°C at pH 6.5 with antibody SAF84 (targeted to the core region of PrP) was found to result in the

production of a diagnostic doublet for ovine BSE of approximately 18kDa and 17kDa, and a single band of approximately 18kDa apparent molecular weight for ovine scrapie (figure 3.4).

As was noted for bromelain, the observed differences between the three prion strains following digestion with protease type XIV were subtle, and while both bromelain and protease type XIV had potential for further investigation as the basis for a strain typing assay it was felt that reliably differentiating small differences in molecular weight between the potentially large number of field- and experimental TSE samples to be examined upon the development of a reliable strain typing assay would prove difficult. Therefore further optimisation was not carried out using these two proteases and it was decided to concentrate on thermolysin as this had indicated that, in contrast to PK, bromelain and protease type XIV, digestion of CNS tissue resulted in the apparent generation of full-length PrP^{Sc} on Western blot.

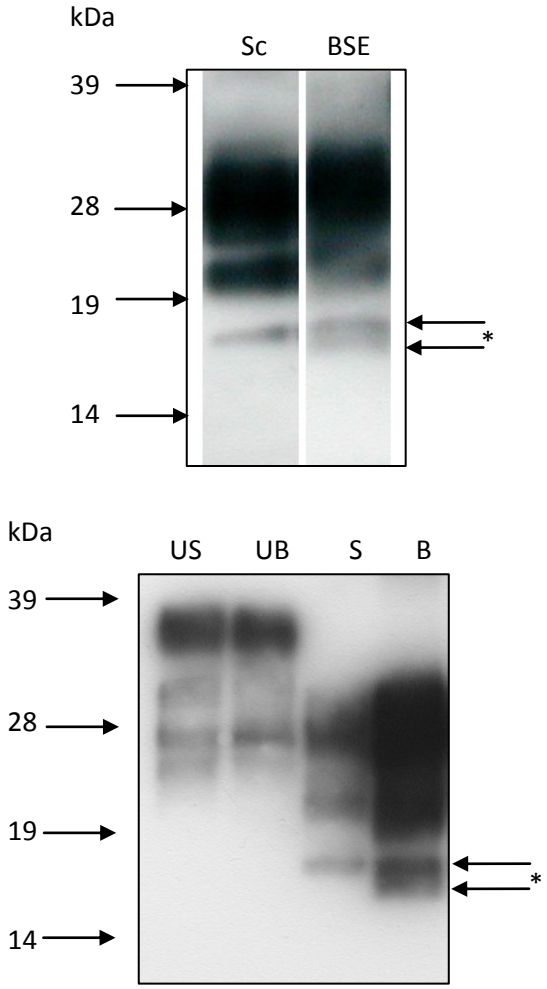


Figure 3.4. Digestion of ovine scrapie, bovine BSE, and ovine BSE brain homogenates with protease XIV. Upper panel: 10% (w/v) brain homogenates of ovine scrapie animal 0575/00 (Sc) and bovine BSE VLA+B pool (BSE) prepared in BHLB at pH 7.4 were digested with 1.6U/ml (320µg/ml) protease type XIV for 1 hour at 37°C, and 3.3µl of 10% digested sample analysed per lane. PrP^{Sc} was detected with anti-PrP antibody 6H4 diluted 1:25,000. Lower panel: brain homogenates (10% (w/v)) from ovine scrapie animal 0575/00 (S) and ovine BSE animal 1693/03 (B) prepared in 10XP BHLB at pH 7.4 were digested for 1 hour with 16U/ml protease type XIV at 37°C. 3.3ul of 10% (w/v) digested sample was loaded per lane, alongside 3.3ul 2% undigested ovine scrapie (US) and ovine BSE (UB) samples from the same animals on 16% SDS PAGE gels. The position of diagnostic doublet for bovine BSE (upper panel) and ovine BSE (lower panel) are indicated (*) corresponding to approximately 18kDa and 17kDa for upper and lower bands. PrP^{Sc} was detected with anti-PrP antibody SAF84 diluted 1:200 and anti-mouse immunoglobulin-HRP conjugated antibody diluted 1:2,000. Signals were visualised using BM chemiluminescence blotting substrate.

3.2.6. The use of the protease thermolysin as the basis for a strain typing assay

While both bromelain and protease type XIV had been shown to be capable of differentiating different prion strains, the subtle differences noted previously were not thought to be robust enough for strain typing purposes. Therefore further investigation was made into using thermolysin as the basis of a strain typing assay. To this end a number of different variables were examined for their effect on the proteolytic activity of thermolysin on PrP^{Sc} with a view to optimising the digestion conditions such that any strain specific differences could be identified by Western blotting.

3.3.1. Optimisation of thermolysin digestion temperature and inhibition of thermolysin using metalloprotease inhibitors

Initial experiments were performed to determine whether thermolysin activity could be rapidly inhibited using metalloprotease inhibitors and whether this affected subsequent digestion by non-metalloproteases. Protease XIV was selected as the non-metalloprotease for this analysis. Azocoll (azo dye bound to collagen) was used as a marker to monitor protease activity, with dye-release determined spectrophotometrically following digestion at 37°C. Thermolysin digestion of azo-dye impregnated collagen was found to be inhibited with 1mM EDTA as monitored by Azocoll protease activity assay (figure 3.5 panel A), and similar results were obtained with EGTA. Inhibition of thermolysin activity with the metalloprotease inhibitor phosphoramidon was absent at 1µm and incomplete at 10µm.

As digestion of Azocoll collagen with protease XIV was observed to occur in the presence of thermolysin and sufficient EDTA inhibitor to inhibit thermolysin, it suggested that little, if any, inhibitory action of EDTA on protease type XIV was occurring (figure 3.5, panel B). Thermolysin digestion of brain homogenate was subsequently inhibited during experimentation with EDTA concentration in excess of 1mM, and it was expected that EDTA would not have an inhibitory effect on any non-metalloproteases tested during sequential digestion of brain homogenate samples.

Given the optimum temperature for thermolysin proteolytic activity is 70°C (manufacturer's data) an analysis of protein profiles following Coomassie staining of protein gels loaded with ovine scrapie and bovine BSE brain homogenates digested with 50µg/ml thermolysin at either 37°C or 70°C for 1 hour was performed and indicated a reduction in residual protein levels at the elevated temperature (data not shown). Therefore, subsequent digestions using thermolysin were typically performed at 70°C.

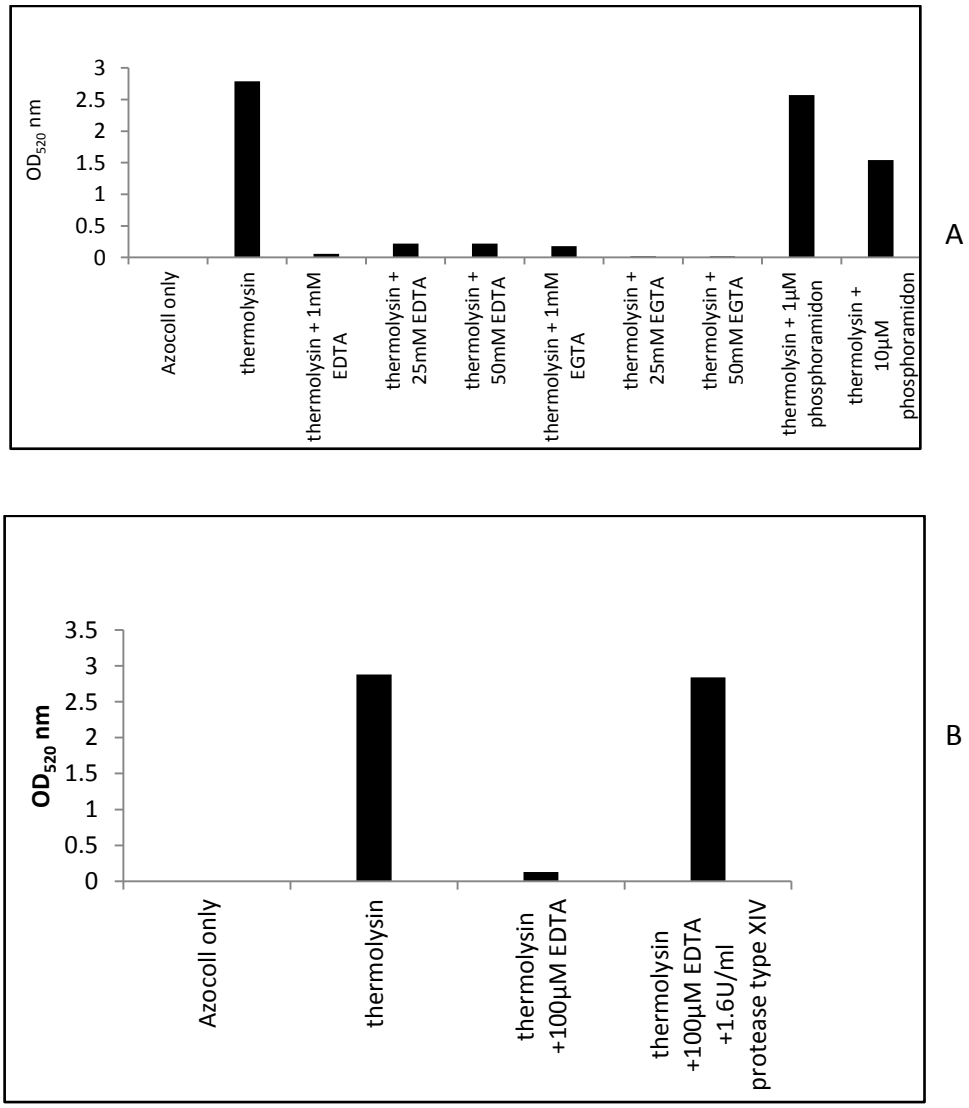


Figure 3.5. Inhibition of thermolysin using EDTA, EGTA, or phosphoramidon. Azo dye impregnated collagen was digested with 50µg/ml thermolysin for 15 minutes at 37°C and proteolysis monitored spectrophotometrically at 520nm (panel A). Inhibition of proteolysis was achieved by pre-incubation of collagen substrate using inhibitor concentrations indicated in panel. Inhibition of thermolysin by pre-incubation of substrate with 100µM EDTA prior to addition of 50µg/ml (1.8U/ml) thermolysin and 1.6U/ml protease type XIV had no effect on protease type XIV (panel B).

3.3.2. Optimisation of thermolysin concentration for the digestion of PrP^C

In order to establish that strain typing profiles from TSE infected ovine and bovine samples were due solely to differences in protease resistant PrP^{Sc} and not due to residual PrP^C, it was necessary to establish digestion conditions which resulted in the complete clearance of PrP^C. Therefore brain homogenates prepared from healthy sheep and cattle were tested against a range of thermolysin and proteinase K concentrations and incubation periods to determine the concentrations of proteases necessary to digest PrP^C.

Initial experiments to optimise the thermolysin concentration necessary to reduce PrP^C below levels detectable by Western blotting indicated differences in the thermolysin susceptibility of ovine and bovine PrP^C (figure 3.6). While incubation for 1 hour at 70°C with 5 µg/ml thermolysin was sufficient to clear PrP^C from ovine brain homogenate, a concentration of 250µg/ml was found necessary to remove bovine PrP^C. It should be noted, however, that much longer exposures of the Western blots were necessary to visualise bovine PrP^C following digestion than would typically be used to visualise undigested PrP^C or thermolysin digested bovine PrP^{Sc} (figure 3.6, panel B1), suggesting that residual levels of bovine PrP^C following thermolysin digestion were very low. At typical exposures used to compare strain specific Western blot banding patterns between ovine scrapie and bovine BSE PrP^{Sc} no residual PrP^C could be detected (figure 3.6, panels A and B). Using these conditions, thermolysin-resistant PrP^{Sc} was readily detected in scrapie infected ovine and BSE infected bovine samples (figure 3.6).

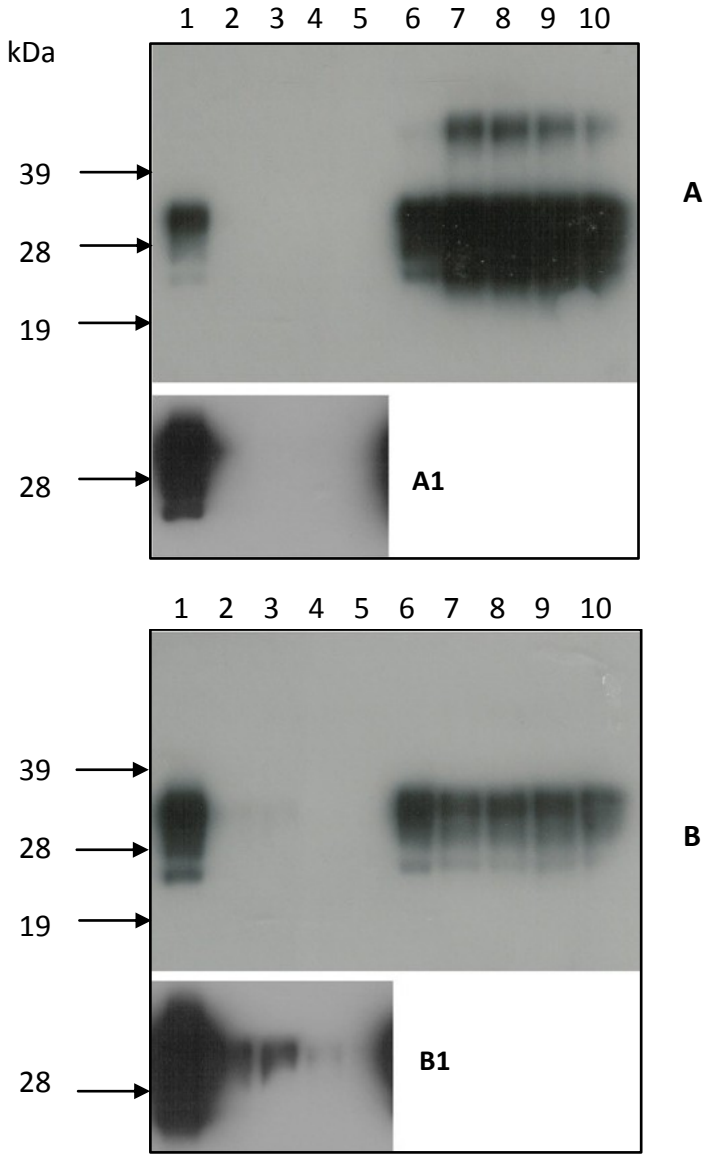


Figure 3.6. Clearance of PrP^C from ovine and bovine brain homogenates using thermolysin. Brain homogenate from healthy (lanes 1-5, VLA-S pool) or TSE infected (lanes 6-10, animal 0575/00) sheep (panels A and A1) were digested with 0, 5, 25, 50, and 100µg/ml thermolysin for 1 hour at 70°C. For undigested samples (lanes 1 and 6), 66µg was loaded per lane (corresponding to 3.3µl of 2% (w/v) brain homogenate). For digested samples (lanes 2-5 and 6-10), 330µg was loaded per lane (corresponding to 3.3µl of 10% (w/v) homogenate). For bovine samples (panels B and B1, pools VLA-B and VLA+B) 0, 25, 50, 100, and 250µg/ml was used. Prolonged exposures of lanes 1-5 are shown in panels A1 and B1, corresponding to ovine and bovine samples respectively. Samples were separated on 12% (w/v) Invitrogen NuPAGE Novex gels and PrP detected with SAF32 diluted 1:2000. Signals were visualised with anti-mouse HRP conjugated secondary antibody in conjunction with BM chemiluminescence blotting substrate.

An analysis of thermolysin digestion of 10% (w/v)healthy ovine and bovine brain homogenates, along with scrapie infected ovine and BSE infected bovine brain homogenates was carried out to determine the overall loss of brain proteins when subsequently analysed by Coomassie staining of SDS-PAGE gels. Data indicated that at concentrations of 5µg/ml (for ovine samples) or 250µg/ml (for bovine samples) thermolysin, digestion resulted in the loss of the vast majority of detectable proteins (figure 3.7).

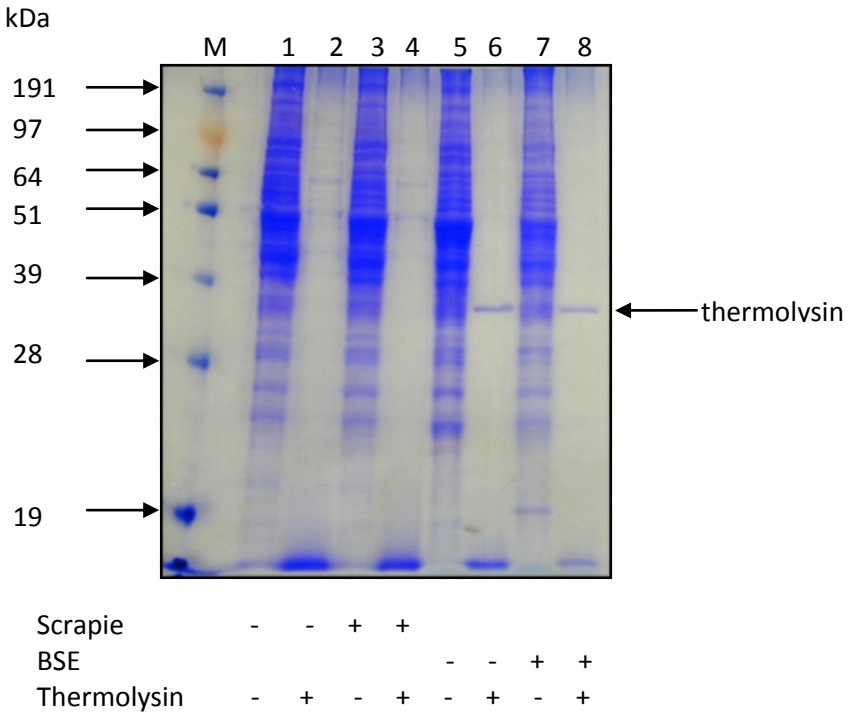


Figure 3.7. Coomassie Blue stained protein gel showing clearance of brain proteins using thermolysin. Ovine (lanes 1-4) or bovine (lanes 5-8) were digested with thermolysin for 1 hour at 70°C. 3.3µl of 10% (w/v) brain homogenates (corresponding to 330µg of wet weight brain material) were digested with 5µg/ml thermolysin (lanes 2 and 4) or 250µg/ml thermolysin (lanes 6 and 8). Healthy ovine (lanes 1-2, VLA-S pool) and scrapie infected ovine (lanes 3-4, animal 0575/00), healthy bovine (lanes 5-6, VLA-B pool) and BSE infected bovine (lanes 7-8, VLA+B pool) were undigested (lanes 1,3,5,7) or digested (lanes 2,4,6,8) as indicated. Samples were separated on 12% (w/v) NuPAGE Novex protein gels and visualised by staining with Coomassie Blue R250.

3.3.3. Clearance of PrP^C from healthy bovine brain homogenates and the generation of protease resistant core PrP^{Sc} from bovine BSE brain homogenates using Proteinase K

Initial experiments had indicated that digestion of brain homogenates from ovine scrapie or bovine BSE infected animals with thermolysin resulted in the production of full-length PrP^{Sc} with a similar Western blot profile to that of PrP^C when detected using an antibody (SAF32) targeted against the N-terminal region of PrP. Clearance of PrP^C from equivalent healthy brain homogenates suggested that Western blot signals were not the result of residual PrP^C. The absence of amino terminal truncation of PrP^{Sc} when digested with thermolysin was in contrast to that observed upon digestion with the archetypal protease used for strain discrimination of TSEs by Western blot, namely proteinase K (figure 3.8). Typically proteinase K digestion results in the loss of amino-terminal epitopes and the generation of a protease-resistant core, with strain differentiation based upon differences in migration profiles (Hope *et al.*, 1999) or loss of amino terminal epitopes (Stack *et al.*, 2002).

Digestion of ovine scrapie and bovine BSE brain homogenates with PK was therefore examined to compare the loss of amino terminal epitopes relative to thermolysin digestion. The persistence of the amino terminal SAF32 epitope upon thermolysin digestion of ovine scrapie and bovine BSE brain homogenates had previously been demonstrated following digestion for 1 hour with 100µg/ml thermolysin at 70°C with no apparent generation of a protease-resistant PrP^{Sc} core (figure 3.6). In contrast, PK digestion of ovine scrapie and bovine BSE infected brain homogenates using 50µg/ml PK at 37°C over a 2 hour time course indicated complete loss of the N-terminal region

of PrP^{Sc} within 15 minutes, as evidenced by the loss of Western blot signal when detected using anti-PrP antibodies AG4 and SAF32 specific to the N-terminal region of PrP (figure 3.8, panels A and B). When probed with an antibody (SAF84) specific to the protease resistant core region of PrP, Western blots demonstrated the typical "triplet" profile of digycosylated, monoglycosylated and unglycosylated PrP^{Sc} (figure 3.8, panel C). The corresponding reduction in apparent molecular weight of PrP^{Sc} following the loss of N-terminal cleavage using PK relative to undigested PrP can be seen (figure 3.8, panel C) and is typical of that previously published e.g. (Collinge *et al.*, 1996).

PK digestion of brain homogenates prepared from healthy animals indicated complete clearance of PrP^{C} following 1 hour incubation at 37°C at 25µg/ml for bovine (figure 3.9) and 10µg/ml PK for ovine brain homogenates (figure 3.10) when detected with anti-PrP antibodies 6H4 and 8G8 respectively, both of which have epitopes within the C-terminal region of PrP. This result is also in agreement with previously published work e.g. (Collinge *et al.*, 1996).

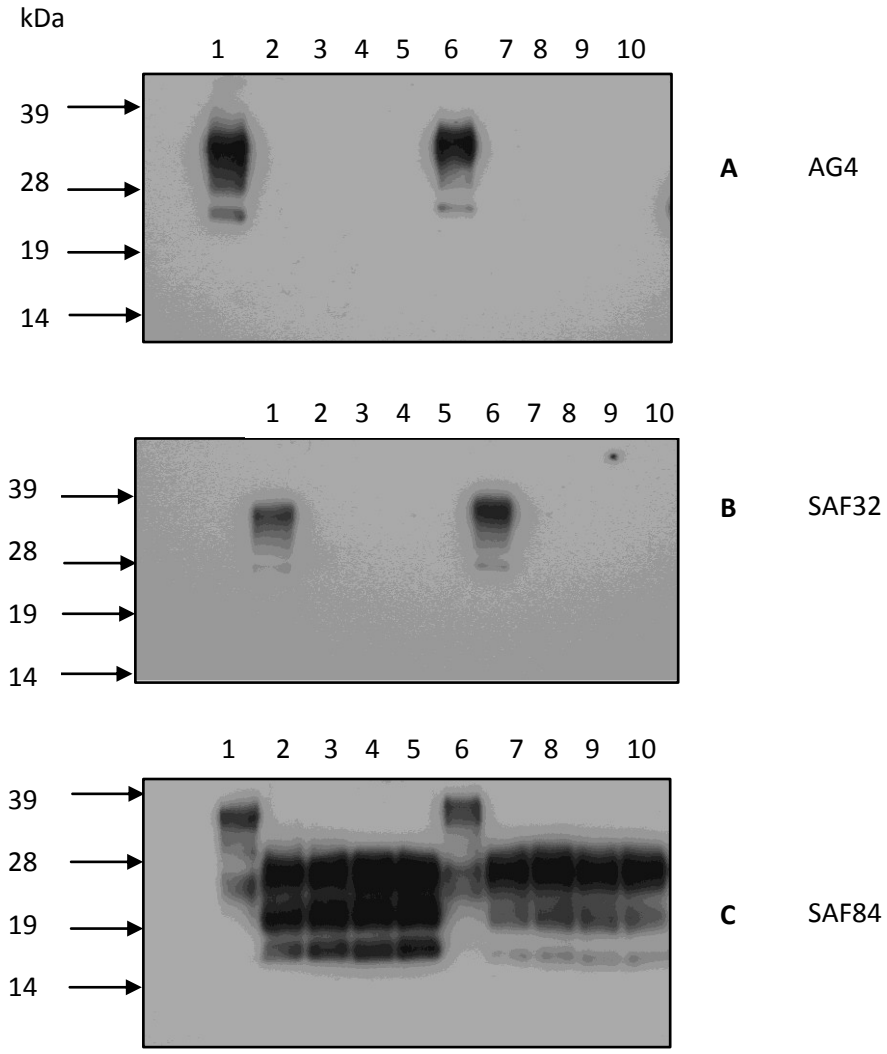


Figure 3.8. Proteinase K digestion of scrapie infected ovine and BSE infected bovine brain homogenates. Brain homogenates (10% (w/v)) prepared in BHLB at pH 7.4 from scrapie infected ovine (lanes 2-5, animal 0575/00) or BSE infected bovine (lanes 7-10, VLA+B pool) were digested for 15, 30, 60, and 120 minutes with 50µg/ml PK at 37°C (lanes 2-5 and 7-10 respectively) and 3.3µl of 10% (w/v) digested homogenate (corresponding to 330µg) loaded per lane on Invitrogen 12% NuPAGE gels. Undigested brain homogenates (3.3µl of 2% (w/v) corresponding to 66µg brain material) were loaded in lanes 1 (ovine) and 6 (bovine). Samples were detected with anti-PrP antibodies AG4 (panel A, diluted 1:4,000), SAF32 (panel B, diluted 1:2,000), or SAF84 (panel C, diluted 1:200). PrP specific antibodies were visualised with HRP conjugated anti-mouse antibody and BM chemiluminescent substrate in conjunction with Biomax MR film.

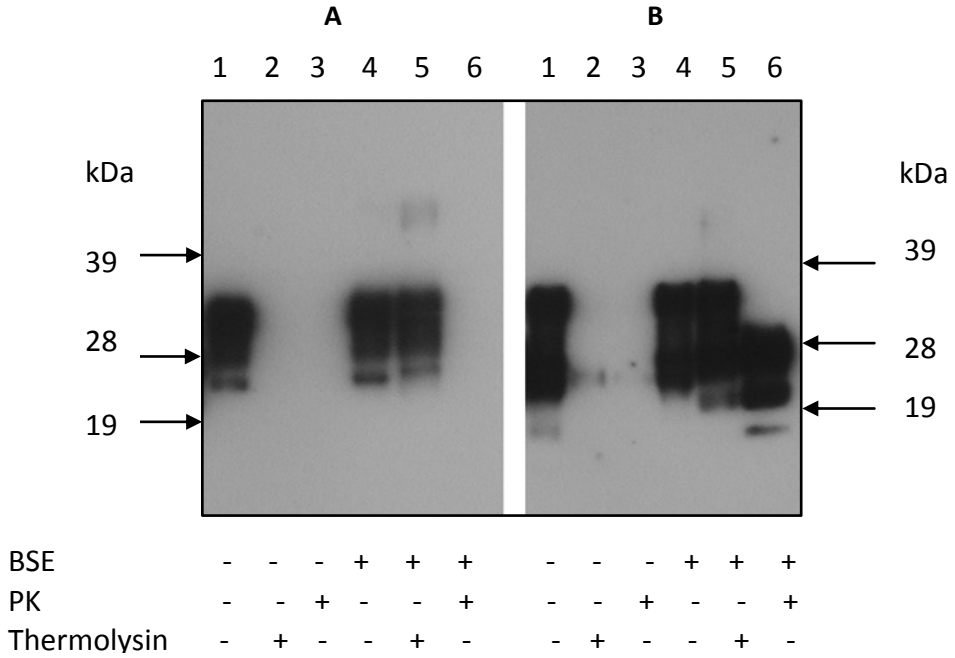


Figure 3.9. Proteinase K and thermolysin digestion of brain homogenates from healthy and BSE infected cattle. Samples of healthy (-, VLA-B pool) and BSE infected bovine (+, VLA+B pool) 10% (w/v) homogenates were digested for 1 hour at 70°C with 250µg/ml thermolysin or 1 hour at 37°C with 25µg/ml PK and 3.3µl 105 (w/v) of digested sample loaded alongside 3.3µl 2% (w/v) undigested sample (lanes 1). Samples were detected with anti-PrP antibody specific to the N-terminal (SAF32) of PrP (panel A) or anti-PrP antibody specific to the C-terminal (6H4) of PrP (panel B). Samples were separated on 12% (w/v) Invitrogen NuPAGE gels and probed with SAF32 diluted 1:2000 (left panel) or 6H4 diluted 1:5000 (right panel), anti-mouse immunoglobulin HRP conjugated antibody, and visualised with BM chemiluminescence blotting substrate.

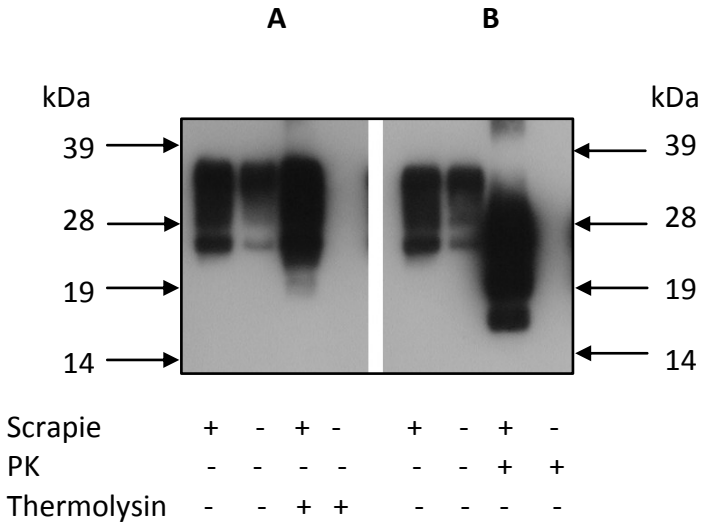


Figure 3.10. Proteinase K and thermolysin digestion of brain homogenates from healthy and scrapie infected sheep. Samples of healthy (-, VLA-S pool) and scrapie infected (+, animal 0575/00) 10% (w/v) homogenates were digested with either 5µg/ml thermolysin for 1 hour at 70°C or 10µg/ml PK for 1 hour at 37°C, and 3.3µl 10% (w/v) digested sample loaded as indicated alongside 3.3µl 2% (w/v) undigested sample. PrP was detected with N-terminal (panel A) or C-terminal (panel B) anti-PrP specific antibodies (SAF32 and 8G8 respectively). Samples were separated on 12% (w/v) NuPAGE gels and PrP detected with antibodies (SAF32 diluted 1:2000 or 8G8 diluted 1:2000), along with anti-mouse immunoglobulin HRP conjugated antibody, and BM chemiluminescence blotting substrate.

3.3.4. Thermolysin digestion of TSE infected brain homogenates

In contrast to the loss of SAF32 epitope seen upon digestion of scrapie and BSE infected brain homogenates with proteinase K, thermolysin digestion resulted in the persistence of the SAF32 epitope and the detection of PrP^{Sc} of a similar size to PrP^{C} as determined by Western blot. Under digestion conditions which resulted in the clearance of all PrP^{C} containing the SAF32 epitope (10µg/ml for ovine and 250ug/ml for bovine), PrP^{Sc} was still readily detected from both BSE infected bovine (figure 3.9) and scrapie infected ovine (figure 3.10) digested samples.

Similarly, using digestion conditions which resulted in the clearance of PrP^{C} from healthy brain homogenates (25µg/ml for bovine and 10µg/ml for ovine), proteinase K digestion of BSE and scrapie infected brain homogenates resulted in the production of PrP^{Sc} fragments detectable by Western blot when detected with antibodies targeted against the PrP27-30 region of PrP, namely antibodies 6H4 and 8G8.

The use of thermolysin for clearance of PrP^{C} and generation of full-length PrP^{Sc} permitted the detection of bovine PrP^{Sc} using either antibodies targeted against the amino terminal or protease resistant core regions of PrP (figures 3.9 and 3.10). This was in contrast to PK digestion, which limited the detection of PrP^{Sc} to antibodies targeted solely against the protease core region of PrP^{Sc} (figure 3.9).

3.3.5. Incubation of TSE infected brain homogenates with sequential additions of thermolysin

Previous work has demonstrated that differential antibody binding as a result of protease digestion can be used to differentiate TSE strains (Stack *et al.*, 2002). The

relative susceptibility of ovine scrapie and bovine BSE PrP^{Sc} to digestion with thermolysin was therefore examined, using antibodies specific to the N-terminal region of PrP (antibodies AG4 and SAF32).

Digestion of 10% (w/v) brain homogenates from scrapie infected sheep and BSE infected cattle with sequential additions of 100µg/ml thermolysin per hour over a 12 hour period indicated considerable differences in protease sensitivity between the two TSE strains when detected with anti-PrP monoclonal antibodies SAF32 or AG4. This data also demonstrated the generation of scrapie specific peptides of lower molecular weight than full length, unglycosylated PrP (figure 3.11).

Detection of PrP^{Sc} with AG4 (which binds N-terminally to the SAF32 binding site (see figure 2.1)) resulted in lower levels of PrP^{Sc} signal compared to SAF32 indicating that cleavage within the AG4 epitope may have been occurring in bovine PrP^{Sc} and, to a lesser extent, in ovine PrP^{Sc}. The increased susceptibility of bovine BSE PrP^{Sc} to digestion compared to ovine PrP^{Sc} resulted in a total loss in Western blot signal after 8 hours digestion when detected with AG4, while scrapie PrP^{Sc} still presented this epitope even after 12 hours of digestion.

The differential susceptibility of PrP^{Sc} from bovine BSE and ovine scrapie brain homogenates to thermolysin digestion, alongside the generation of scrapie-specific peptides not observed in bovine BSE digestions indicated the potential use of a combination of thermolysin and N-terminal PrP-specific antibodies for the production of a Western blot based method capable of strain differentiation.

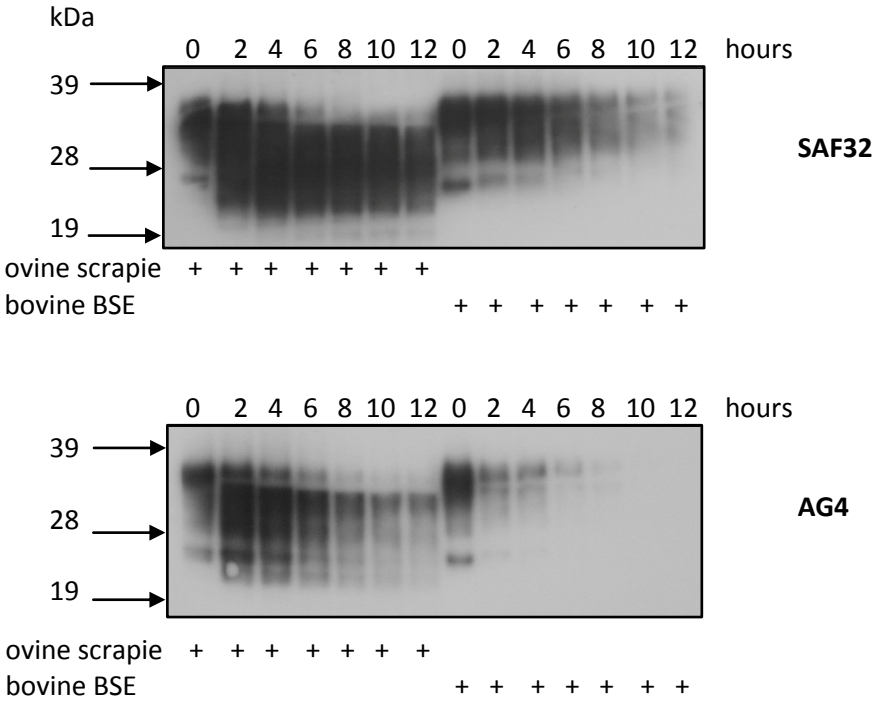


Figure 3.11. Thermolysin digestion of ovine scrapie and bovine BSE brain homogenates over a 12 hour time course. Brain homogenates (3.3µl of 10% (w/v)) from ovine scrapie (animal 0575/00) or bovine BSE (VLA+B pool) were digested with 100µg/ml/hour thermolysin for 12 hours and aliquots removed every 2 hours as indicated. 3.3µl of 2% (w/v) undigested brain homogenates were also loaded (represented by 0 above lane) on 12% Invitrogen NuPAGE gels. PrP was detected with anti-PrP specific monoclonal antibodies SAF32 (diluted 1:2,000) or AG4 (diluted 1:4,000) as indicated and anti-mouse HRP conjugated antibody, and visualised using HRP chemiluminescent substrate.

3.3.6. The effect of pH on thermolysin digestion of PrP^{Sc}

Experiments had indicated that thermolysin digestion of scrapie infected ovine and BSE infected bovine brain homogenates resulted in differences in Western blot profiles (figure 3.11), in that peptides of lower molecular weight than unglycosylated PrP at approximately 19-26 kDa were present in thermolysin digested ovine scrapie samples but absent in bovine BSE samples. This strain dependent Western blot profiling suggested the basis of a novel assay based on the production of thermolysin-resistant PrP^{Sc} and its detection using antibodies specific to the N-terminal region of PrP. To this end, attempts were made to optimise the digestion of brain homogenates in order to optimise the differences observed and produce a robust strain typing assay.

Digestion of 10% (w/v) ovine scrapie brain homogenate 0575/00 and 10% (w/v) VLA+ B pool of bovine brain homogenate were prepared at pH values ranging from pH 6.5 to pH to 8.5 using BHLB with elevated phosphate levels as described in section 2.2.1. Samples were digested by the sequential addition of 100µg/ml/hour thermolysin for 4 hours which indicated that at pH values greater than pH 6.5 ovine scrapie brain homogenate generated an increased proportion of SAF32 immuno-reactive peptides in the range of 19kDa to 26kDa relative to the equivalent bovine BSE sample (figure 3.12). No increase in production of these peptides was detected from the corresponding bovine BSE brain homogenate across the pH range of 6.5 to 8.5, again suggesting that generation of these peptide fragments was a strain-associated phenomenon.

However, thermolysin digestion of ovine scrapie samples resulted in an overall qualitative increase of SAF32 reactive PrP^{Sc} as pH increased, suggesting a possible reduction in digestion efficiency of ovine scrapie PrP^{Sc} at increasing pH. Notably, a similar increase in total bovine PrP^{Sc} levels was not observed. These data suggest that although pH was having an effect on the susceptibility of ovine PrP^{Sc} to thermolysin digestion, it did not result in a significant increase in the generation of strain specific fragments. Brain homogenates were therefore subsequently prepared at pH 7.4 using BHLB containing 10X the standard phosphate concentration to minimise pH variation between brain homogenate samples.

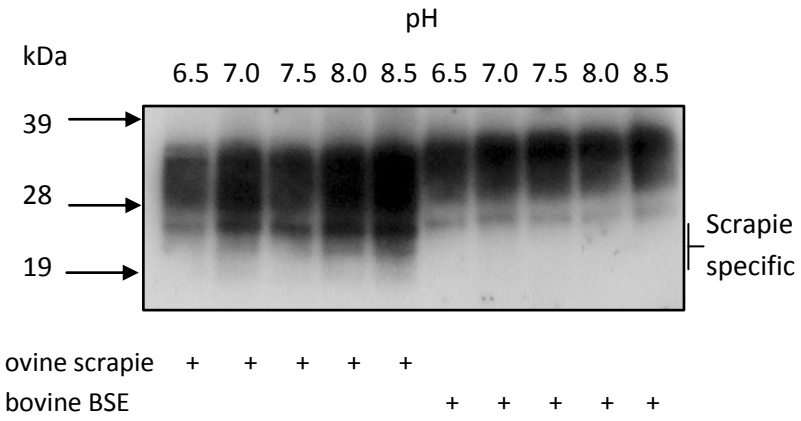


Figure 3.12. The generation of N-terminal SAF32 immunoreactive peptides from ovine scrapie brain homogenate at increasing pH values. Ovine scrapie brain homogenate 0575/00 and bovine BSE brain homogenate VLA+B pool prepared at the indicated pH were digested for 4 hours with 100µg/ml/hour thermolysin and 3.3µl 10% digested homogenate loaded per well on Invitrogen 12% NuPAGE gels. Scrapie specific PrP peptide fragments are shown as indicated. PrP^{Sc} was detected with anti-PrP monoclonal antibody SAF32 diluted 1:2000 and visualised using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

3.3.7. Thermolysin digestion of ovine scrapie and ovine BSE brain homogenates

The use of limited proteolysis with thermolysin as a method to differentiate ovine scrapie PrP^{Sc} from bovine PrP^{Sc} had indicated that a combination of sequential additions of thermolysin to brain homogenate and the detection of the resulting PrP^{Sc} fragments by Western blot using antibodies targeted to the N-terminal of PrP was capable of differentiating two TSE strains in different host species, namely sheep and cattle. A pre-requisite of the assay however was deemed to be the capability to differentiate two TSE strains in a single host species. The absence of differences in the primary sequence of PrP for both ovine scrapie and ovine BSE (both animals were ARQ/ARQ genotype for PrP) would imply that any strain differentiation observed following thermolysin digestion and Western blot analysis would be due to the conformational differences in PrP^{Sc} strains influencing protease accessibility. To this end, the strain typing assay was optimised for the differentiation of scrapie and BSE in an ovine host.

An initial analysis of thermolysin digestion in conjunction with SAF32 detection by Western blot of ovine scrapie 0575/00 (genotype ARQ/ARQ), bovine BSE, and ovine BSE PrP^{Sc} 0394/04 (genotype ARQ/ARQ) homogenates following sequential digestion with 100µg/ml per hour thermolysin indicated the presence of 19-26kDa SAF32 reactive peptide fragments from ovine scrapie and to a lesser extent from ovine BSE, and an absence from the corresponding bovine BSE sample (figure 3.13). As the ovine samples were of an identical prion genotype, these observed differences were therefore not due to differences in primary amino acid sequence resulting in differential protease susceptibility. Previous work by Stack and colleagues had

indicated that anti-PrP monoclonal antibody P4 was capable of differentiating ovine BSE from ovine scrapie when used in conjunction with limited PK digestion (Stack *et al.*, 2002). Therefore experimental analyses were performed using thermolysin digestion in conjunction with P4 detection by Western blot.

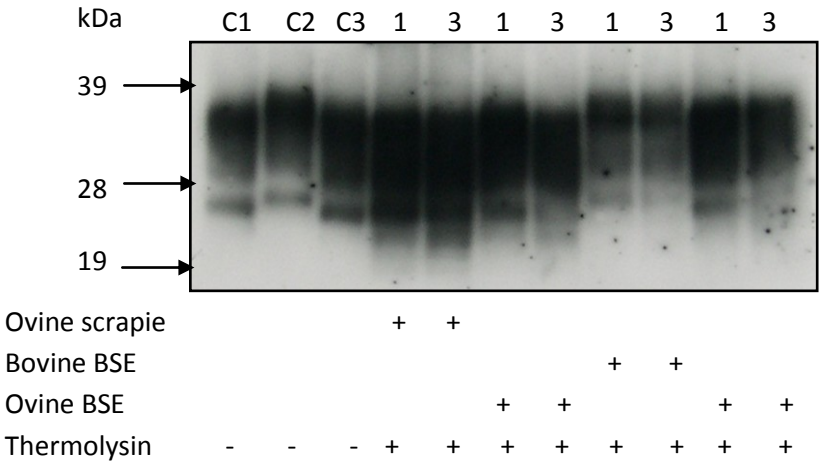


Figure 3.13. Sequential digestion of ovine scrapie, bovine BSE, and ovine BSE brain homogenates for 3 hours with 100µg/ml thermolysin per hour. Undigested ovine scrapie 0575/00 (C1), bovine BSE VLA+B pool (C2), and ovine BSE 0394/04 (C3), and thermolysin digested for 1 or 3 hours at 100µg/ml/hour where indicated were analysed by Western blot. Undigested (3.3µl of 2% (w/v)) or thermolysin digested (3.3µl 10% (w/v)) were loaded per well in Invitrogen 12% NuPAGE gels. Following transfer to PVDF, PrP was detected using anti-PrP monoclonal antibody SAF32 diluted 1:2000 and visualised using anti-mouse HRP conjugated antibody and HRP chemiluminescent substrate.

A comparative digestion of ovine scrapie 0575/00 brain homogenate with BSE ovine brain homogenate (animal 0394/04) at both pH 6.5 and pH 7.5 with 100µg/ml/hour thermolysin for 4 and 8 hours followed by Western blotting of the samples and detection with anti-PrP antibody P4 was performed. Data suggested that at pH 7.5 there were two markers capable of distinguishing scrapie from BSE in the same host species, notably the generation of scrapie specific peptides in the region of 19kDa to 26kDa, and a differential susceptibility to thermolysin digestion (figure 3.14) after 8 hours sequential digestion. Confirmation of this result using a time course of 1, 4, 6, and 8 hours sequential thermolysin digestion at 100µg/ml/hour followed by Western blotting and detection with SAF32 and P4 indicated that a combination of thermolysin digestion at a controlled pH followed by detection using antibodies specifically targeted to the N-terminal region of the prion protein was capable of distinguishing two TSE strains in the same host species (figure 3.15). Notable was the increased susceptibility of ovine BSE PrP^{Sc} compared to ovine scrapie PrP^{Sc} to degradation by thermolysin at a region covering the P4 epitope. These data suggest that this differential susceptibility was as a result of strain-encoded conformational differences affecting protease accessibility, and not differences in the primary PrP sequence or antibody affinity as could be hypothesised for the differential susceptibility to digestion between ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc}. Finally, this work suggests that the use of thermolysin complements previously published observations that monoclonal antibody P4 in conjunction with limited proteolysis with proteinase K is capable of differentiating ovine scrapie from ovine BSE (Stack *et al.*, 2002).

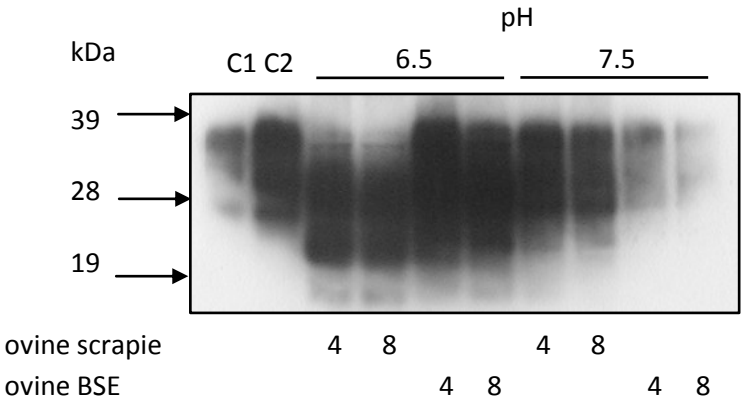


Figure 3.14. Digestion of ovine scrapie and ovine BSE brain homogenates at pH 6.5 and pH 7.5 for 4 and 8 hours with sequential additions of 100µg/ml thermolysin per hour. Undigested ovine scrapie (animal ID 0575/00) at pH 6.5 (C1), ovine BSE (animal ID 0394/04) at pH 6.5 (C2), and thermolysin digested ovine scrapie and ovine BSE at pH 6.5 and 7.5 were digested for 4 or 8 hours (where indicated) with 100g/ml/hour thermolysin and analysed by Western blotting. 3.3µl of 10% (w/v) digested brain homogenate along with 3.3µl of 2% (w/v) undigested samples from both homogenates were loaded per well in Invitrogen NuPAGE 12% SDS PAGE gels. Following Western blotting to PVDF, PrP was detected using anti-PrP monoclonal antibody P4 diluted 1:10 and visualised using anti-mouse HRP conjugated antibody and HRP chemiluminescent substrate.

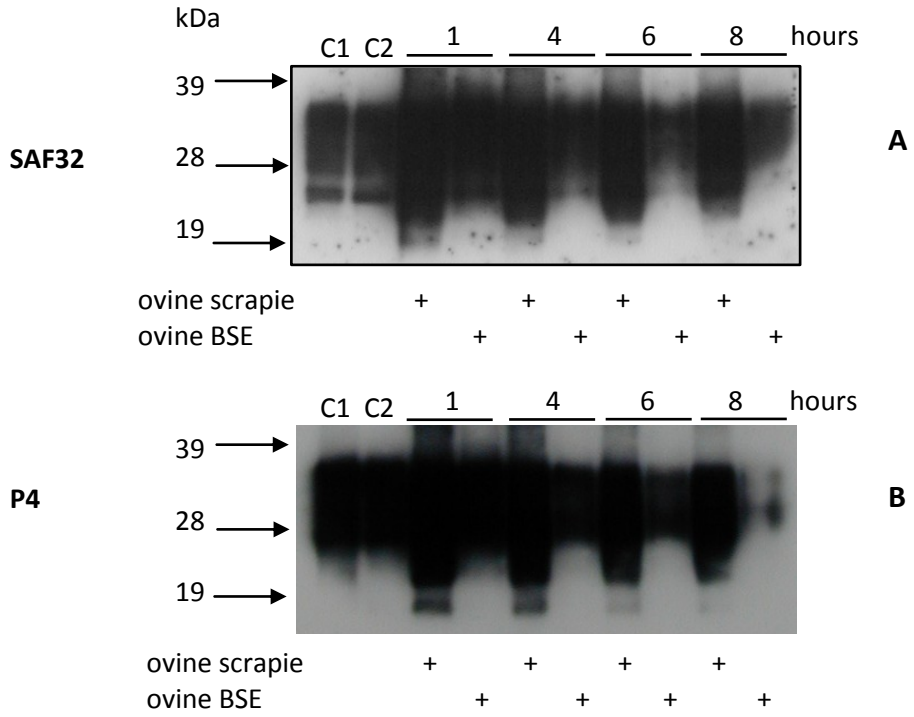


Figure 3.15. Sequential digestion of ovine scrapie and ovine BSE 10% (w/v) brain homogenates at pH 7.5 for 1, 4, 6, and 8 hours with 100µg/ml/hour thermolysin at 70°C and detected with PrP specific antibodies SAF32 and P4. 6.6µl of 10% (w/v) of each digested homogenate, along with 6.6µl of 2% undigested ovine scrapie (C1, animal ID 0575/00)) homogenate at pH 7.5 and 6.6µl of 2% undigested ovine BSE (C2, animal ID 0394/04) homogenate at pH 7.5 were loaded per well on Invitrogen 12% NuPAGEgels. Proteins were transferred to PVDF membrane and detected using PrP specific antibodies SAF32 (panel A, diluted 1:2,000) and P4 (panel B, diluted 1:10). Signals were visualised using HRP chemiluminescent substrate following detection of PrP antibodies using mouse specific HRP conjugated antibody.

CHAPTER 4

Application of a thermolysin strain-typing assay to ovine scrapie field cases, experimental ovine BSE and experimental ovine scrapie strains CH1641 and SSBP/1, and the endogenous cleavage of PrP within the CNS of TSE-affected sheep

4.1 Introduction

This chapter describes the application of the novel strain typing test developed in chapter 3 to a range of CNS tissues from ovine scrapie field cases, experimental ovine scrapie strains, and experimental ovine BSE cases. The application of the assay to a range of cases such as this was performed to provide data on the robustness of the assay when tested against a wider range of samples and PrP genotypes than was available during the development of the test. Data generated on the neuroanatomical distribution of PrP^{Sc} using the test in conjunction with endogenously produced PrP peptide fragments in the available samples was used to potentially identify scrapie strains.

4.2.1 Application of thermolysin strain typing assay to field cases of ovine scrapie and experimental BSE

The thermolysin strain typing assay had been demonstrated to be capable of distinguishing PrP^{Sc} from three TSE strains, namely ovine scrapie (ARQ/ARQ genotype), ovine BSE (ARQ/ARQ genotype) and bovine BSE. Following the very limited number of samples and genotypes used to optimise the strain typing assay, a number of further samples were analysed to confirm that the assay was reproducible with a wider range of experimental ovine BSE animals and field cases of ovine scrapie. This was particularly important as the latter samples would cover a range of breeds, genotypes and potentially strains. The molecular profiles of experimental scrapie strains following thermolysin digestion were also investigated using strain CH1641 and SSBP/1. Finally, the distribution and protein profiles of thermolysin-resistant PrP^{Sc} was

investigated in three CNS regions (namely caudal medulla, cerebellum, and spinal cord) from the ovine scrapie field cases, permitting an analysis of the neuroanatomical distribution of thermolysin-resistant PrP^{Sc}.

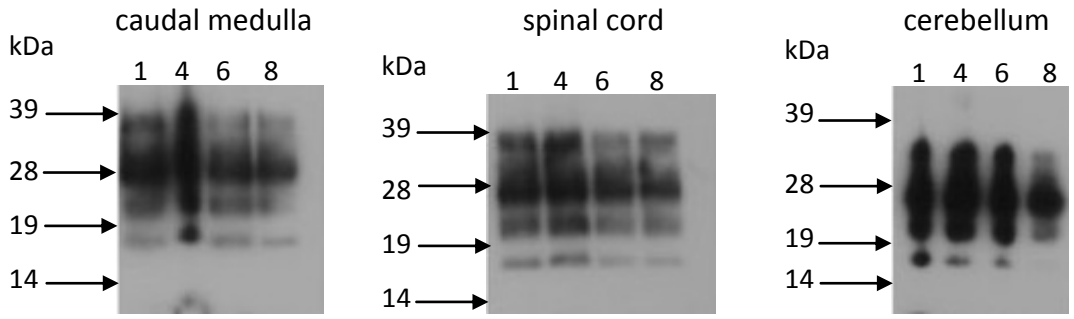
CNS material consisting of brain stem, cerebellum, and spinal cord from a number of sheep collected as part of the UK Government's scrapie surveillance program were analysed for strain characteristics using the thermolysin assay. Details of the animal genotypes and other relevant data are given in table 2.1. Control samples consisting of experimental scrapie strains CH1641 and SSBP/1 were also analysed, along with sheep experimentally infected with BSE. Experimental ovine BSE and bovine BSE cases were included in the analysis, although the more limited distribution of PrP^{Sc} within bovine BSE brain and the limited availability of certain specific ovine BSE CNS tissues precluded the examination of thermolysin-resistant PrP^{Sc} in certain CNS regions.

4.2.2 Detection of thermolysin-resistant PrP^{Sc} from field cases of ovine scrapie and experimentally infected ovine BSE

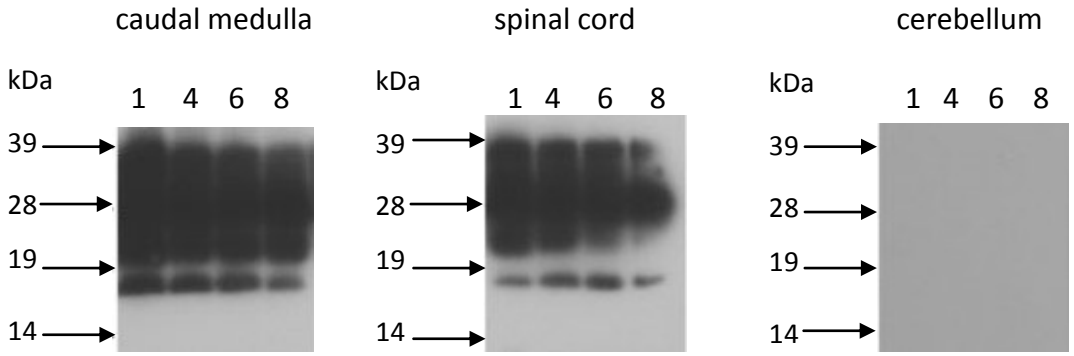
Fifteen natural scrapie cases were analysed covering a range of breeds and genotypes and distinct CNS regions. Alongside these natural scrapie cases, 4 experimental ovine BSE cases of genotype ARQ/ARQ were also analysed using the CNS regions available. Where possible analyses were performed on the caudal medulla, cerebellum, and C1-C2 spinal cord, however the limited availability of certain tissues limited analysis of ovine BSE cases to the caudal medulla and cerebellum regions only, with 4 further scrapie cases (animal 0575/00, 0210/03, 0635/03 and 0678/03) limited to either the caudal medulla region alone or caudal medulla and cerebellum (as indicated in figures 4.1 to 4.6). A concentration of 150µg/ml thermolysin was used for the analyses due to an apparent batch-to-batch variation in thermolysin activity which resulted in the incomplete clearance of PrP^{C} from healthy CNS samples when digested with 100µg/ml thermolysin under standard conditions.

Following 8 hours sequential digestion, Western blots of caudal medulla homogenate digests indicated P4-reactive peptide bands of approximately 19kDa, 23kDa, 28kDa, and 36kDa in scrapie samples, while ovine BSE samples only produced poorly resolved bands at 28kDa and 36kDa. These results were consistent for four experimental ovine BSE animals of PrP genotype ARQ/ARQ as well as the 15 scrapie field isolates with a range of PrP genotypes, namely ARQ/ARQ, VRQ/VRQ, ARQ/VRQ, and AHQ/AHQ, for which caudal medulla tissue was available for analysis. For all of these animals, neither PrP genotype nor breed appeared to influence the presence of strain-specific bands (figures 4.1 to 4.6).

Ovine scrapie 0615/03 (ARQ/ARQ)



Ovine scrapie 0456/03 (ARQ/VRQ)



Ovine scrapie 0455/03 (VRQ/VRQ)

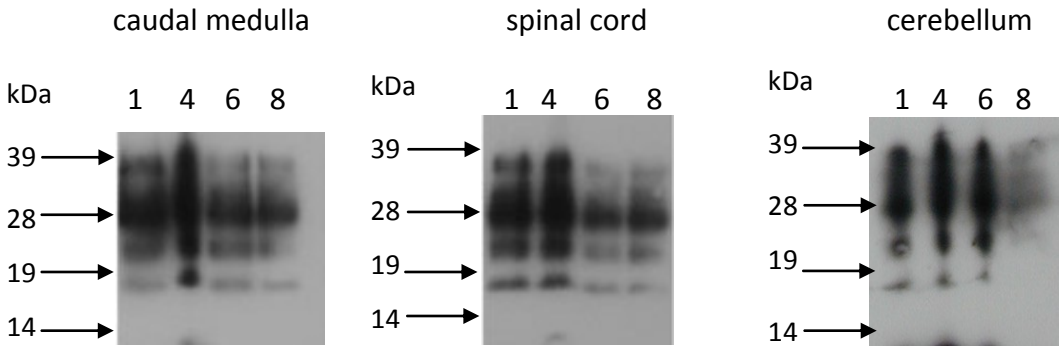
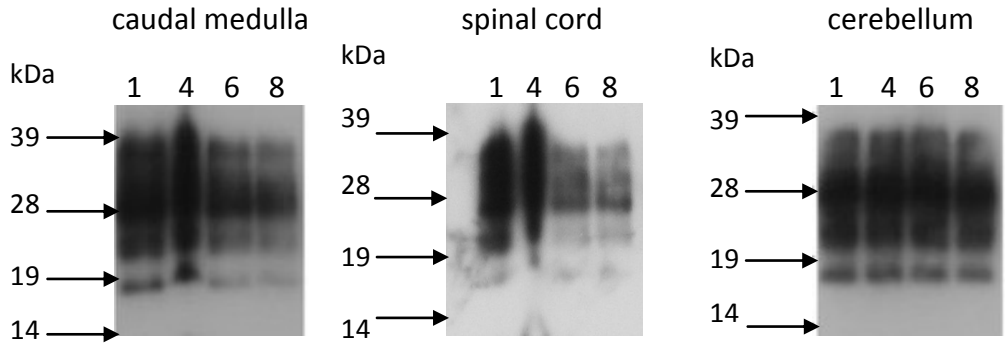
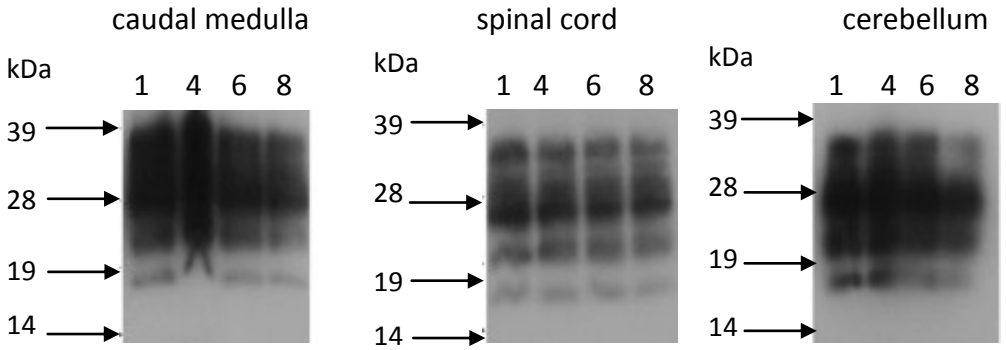


Figure 4.1 Analysis of three CNS regions from scrapie infected sheep 0615/03, 0456/03, and 0455/03. 10% (w/v) homogenates from caudal medulla, cerebellum, and C1-C2 spinal cord regions were digested with 150µg/ml/hour thermolysin for 1 hour (lane labelled 1), 4 hours (lane labelled 4), 6 hours (lane labelled 6) and 8 hours (lane labelled 8) prior to detection with anti-PrP antibody P4 diluted 1:1000. Signals were detected using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

Ovine scrapie 0836/03 (ARQ/VRQ)



Ovine scrapie 0923/03 (VRQ/VRQ)



Ovine scrapie 0925/03 (VRQ/VRQ)

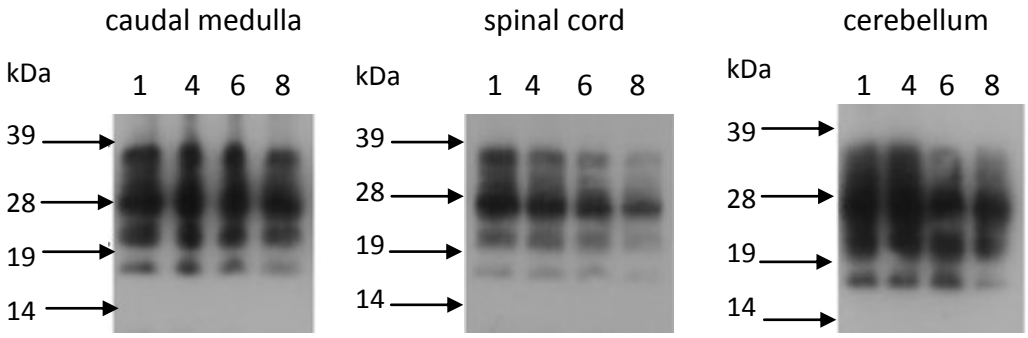
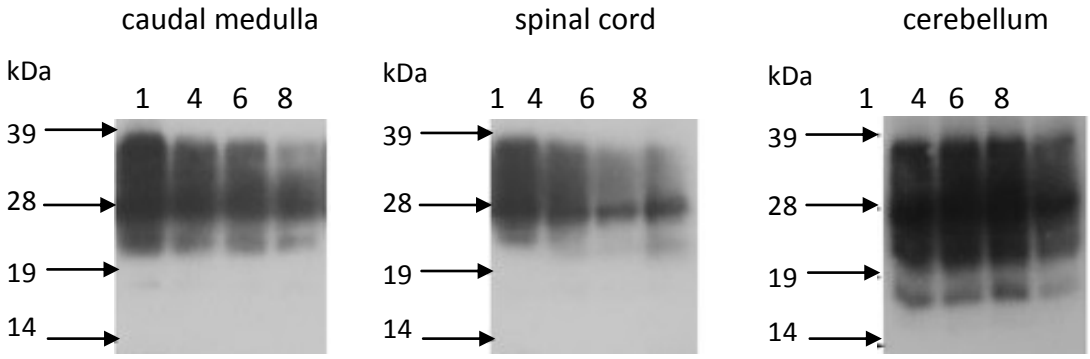
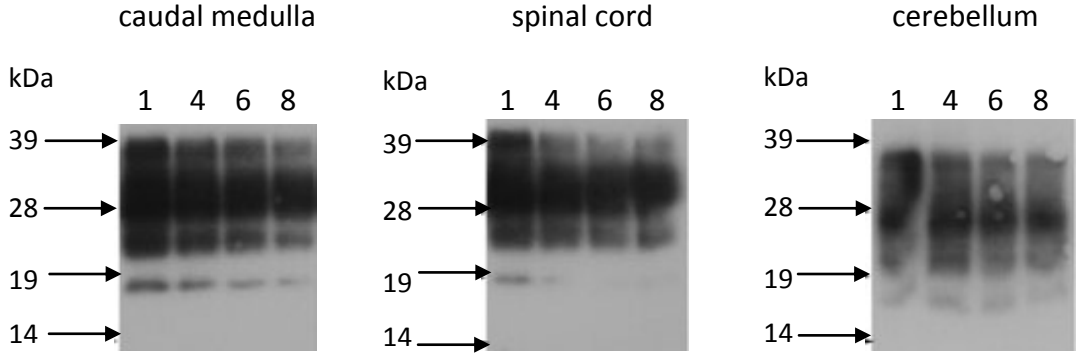


Figure 4.2 Analysis of three CNS regions from scrapie infected sheep 0836/03, 0923/03, and 0925/03. 10% (w/v) homogenates from caudal medulla, cerebellum, and C1-C2 spinal cord regions were digested with 150µg/ml/hour thermolysin for 1 hour (lane labelled 1), 4 hours (lane labelled 4), 6 hours (lane labelled 6) and 8 hours (lane labelled 8) prior to detection with anti-PrP antibody P4 diluted 1:1000. Signals were detected using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

Ovine scrapie 1563/02 (VRQ/VRQ)



Ovine scrapie 1276/02 (VRQ/VRQ)



Ovine scrapie 1275/02 (VRQ/VRQ)

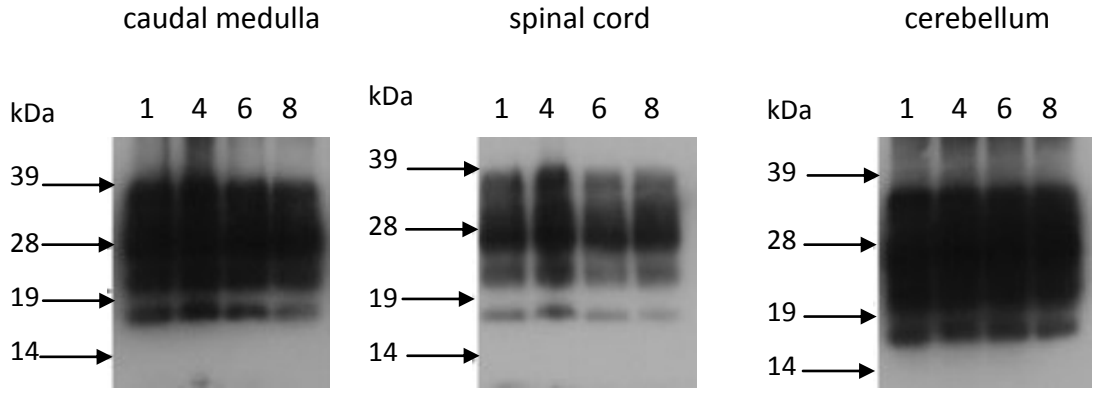
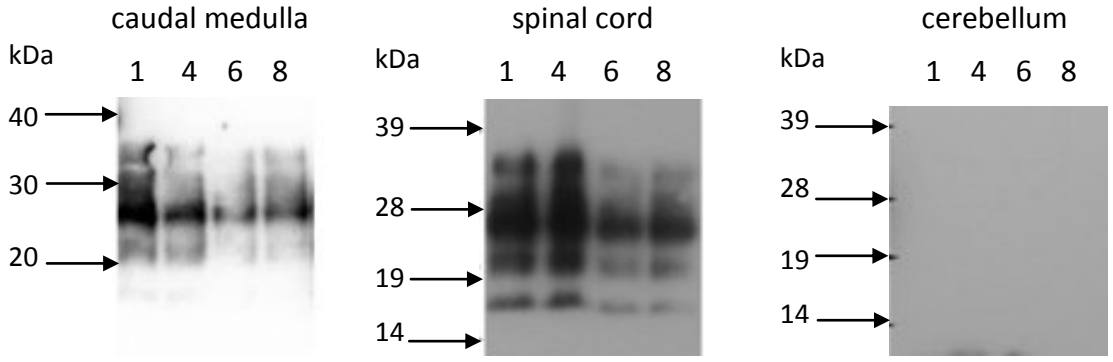
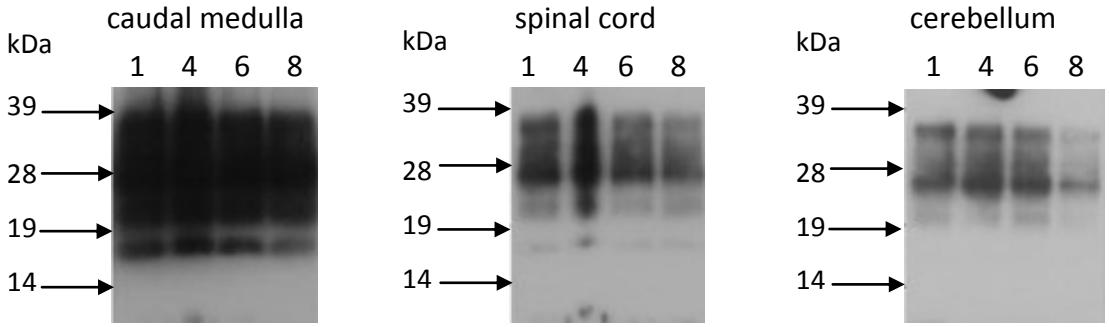


Figure 4.3 Analysis of three CNS regions from scrapie infected sheep 1563/02, 1276/02, and 1275/02. 10% (w/v) homogenates from caudal medulla, cerebellum, and C1-C2 spinal cord regions were digested with 150µg/ml/hour thermolysin for 1 hour (lane labelled 1), 4 hours (lane labelled 4), 6 hours (lane labelled 6) and 8 hours (lane labelled 8) prior to detection with anti-PrP antibody P4 diluted 1:1000. Signals were detected using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

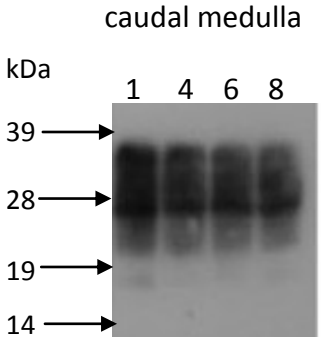
Ovine scrapie 0284/97 (AHQ/AHQ)



Ovine scrapie 0226/03 (ARQ/VRQ)



Ovine scrapie 0575/00 (ARQ/ARQ)



Ovine BSE 0394/04 (ARQ/ARQ)

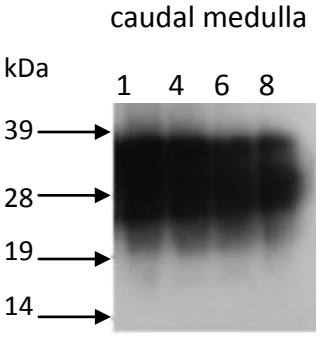


Figure 4.4 Analysis of three CNS regions from scrapie infected sheep 0284/97 and 0226/03, caudal medulla from scrapie infected sheep 0575/00, and caudal medulla from BSE infected sheep 0394/04. 10% (w/v) homogenates from caudal medulla, cerebellum, and C1-C2 spinal cord regions were digested with 150µg/ml/hour thermolysin for 1 hour (lane labelled 1), 4 hours (lane labelled 4), 6 hours (lane labelled 6) and 8 hours (lane labelled 8) prior to detection with anti-PrP antibody P4 diluted 1:1000. Signals were detected using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

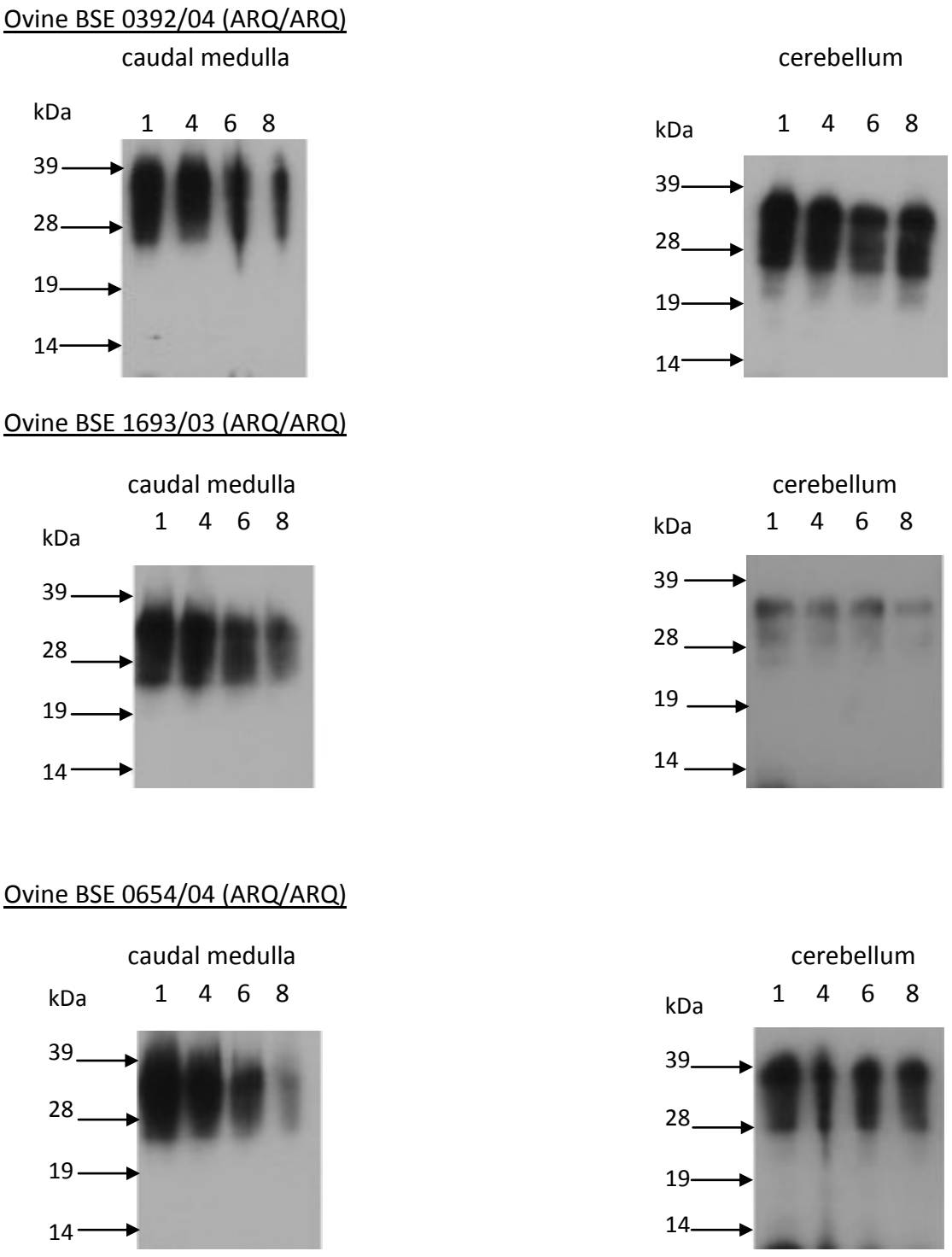


Figure 4.5 Analysis of two brain regions from BSE infected sheep 0392/04, 1693/03 and 0654/04. 10% (w/v) homogenates of caudal medulla and cerebellum were digested with 150µg/ml/hour thermolysin for 1 hour (lane labelled 1), 4 hours (lane labelled 4), 6 hours (lane labelled 6) and 8 hours (lane labelled 8) prior to detection with anti-PrP antibody P4 diluted 1:1000. Signals were detected using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

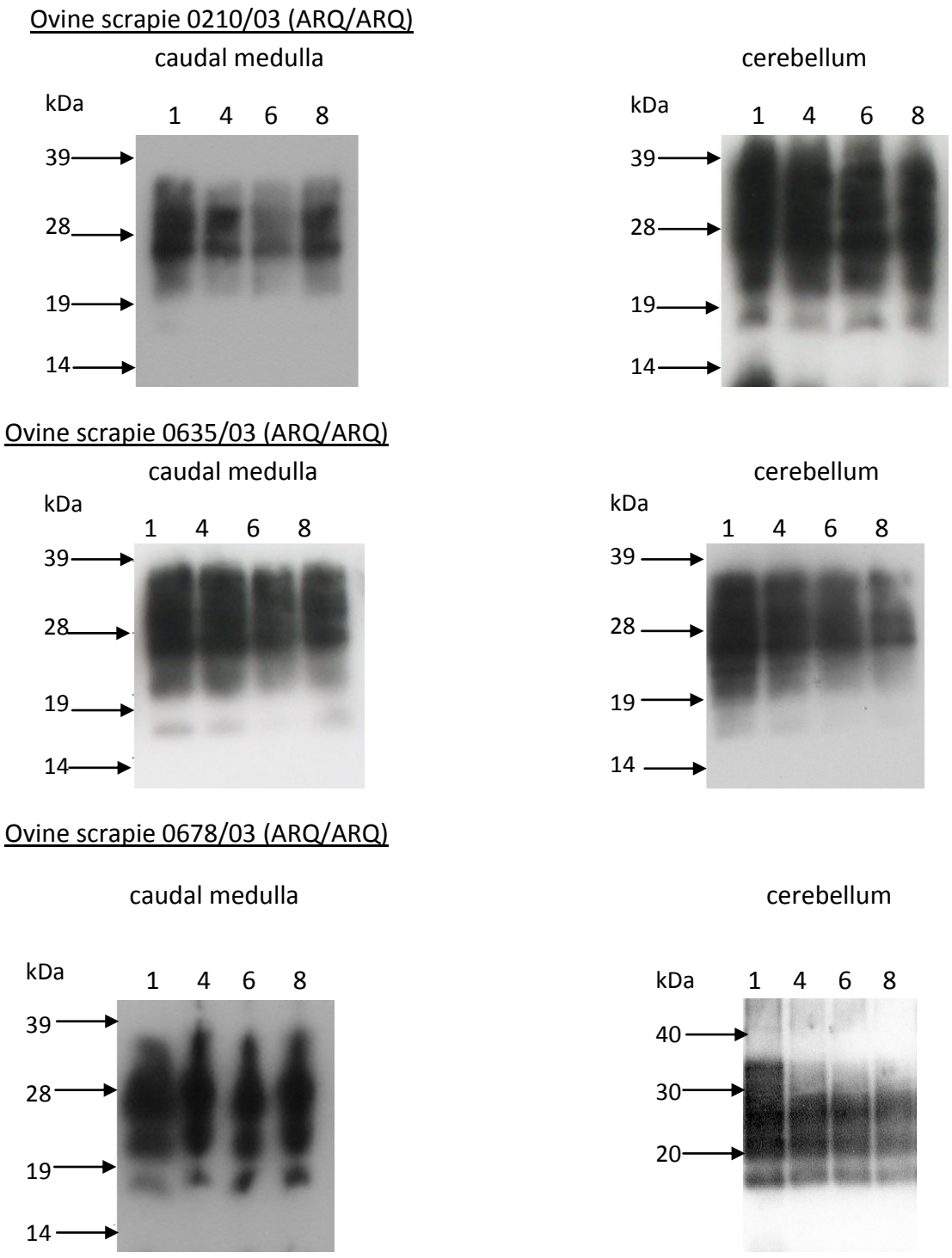


Figure 4.6 Analysis of caudal medulla and cerebellum from scrapie infected sheep 0210/03, 0635/03, and 0678/03. 10% (w/v) homogenates were digested with 150µg/ml/hour thermolysin for 1 hour (lane labelled 1), 4 hours (lane labelled 4), 6 hours (lane labelled 6) and 8 hours (lane labelled 8) prior to detection with anti-PrP antibody P4 diluted 1:1000. Signals were detected using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

4.2.3 Deposition of thermolysin-resistant PrP in three CNS regions of TSE affected sheep

The application of the thermolysin strain typing assay was applied to homogenates prepared from caudal medulla, spinal cord, and/or cerebellum regions of TSE affected sheep in order to determine the distribution of thermolysin-resistant PrP^{Sc} within the CNS regions of these animals and to determine whether the thermolysin assay gave consistent results in each of the CNS regions analysed.

As described previously, deposition of thermolysin-resistant PrP^{Sc} within the caudal medulla region of the ovine scrapie cases was observed in all ovine cases following both 1 and 8 hours digestion with 150µg/ml thermolysin. Analysis of spinal cord homogenates from 11 animals for which this tissue was available indicated the presence of protease resistant PrP^{Sc} in all spinal cord samples following 1 hour and 8 hours digestion with 150µg/ml thermolysin (figure 4.1 to 4.4). The banding pattern of thermolysin-resistant PrP^{Sc} was typical of that observed for caudal medulla samples.

In contrast to the presence of protease resistant PrP^{Sc} following 1 hour digestion with 150µg/ml thermolysin seen in all 11 ovine caudal medulla and spinal cord samples, deposition of thermolysin-resistant PrP^{Sc} within the cerebellum of ovine scrapie samples was observed in only 12 of 14 cases for which cerebellum tissue was available (figures 4.1 to 4.6). Two animals (0284/97 and 0465/03) indicated an absence of detectable PrP^{Sc} after a single hour's digestion with this level of protease (figures 4.1 and 4.4). Protease resistant PrP^{Sc} was also readily detected in cerebellum from these 12 animals following 8 hours digestion with 150µg/ml/hour thermolysin with the

further exception of one animal (0455/03) which displayed relatively low levels of protease resistant PrP^{Sc} at this time point and poor resolution of the sample.

Detection of thermolysin-resistant PrP^{Sc} in 10% (w/v) cerebellum homogenates from animals 0284/97 and 0456/03 using an anti-PrP antibody L42 specific to the protease resistant core of PrP^{Sc} confirmed the absence of thermolysin-resistant PrP^{Sc} (figure 4.7, panels A and B) and not simply loss of the N-terminally located P4 epitope.

Digestion of caudal medulla brain and cerebellum homogenates with 50µg/ml proteinase K for 1 hour further confirmed the absence of PrP^{Sc} within the cerebellum of animals 0284/97 and 0456/03 (figure 4.7, panel D and E). This is in contrast to, for example, animal 1276/02 where both PK-resistant and thermolysin-resistant PrP^{Sc} were detected within the cerebellum (figure 4.8, panel C).

For ovine BSE samples (for which no spinal cord tissue was available) the caudal medulla regions produced identical BSE-specific PrP^{Sc} profiles typical of those observed previously (figure 4.5). Thermolysin resistant PrP^{Sc} was detected in the corresponding cerebellum samples, although a single animal (1693/03) demonstrated reduced levels of PrP^{Sc} in the cerebellum relative to the caudal medulla.

Overall, only the caudal medulla region gave clear resolution of the thermolysin-resistant PrP^{Sc} profile and a relatively high level of PrP^{Sc} for all isolates. When high levels of PrP^{Sc} were detected in the spinal cord or cerebellum of isolates the thermolysin-resistant PrP^{Sc} profile was identical to that for caudal medulla, while cerebellum from 2 isolates gave no thermolysin-resistant PrP^{Sc}.

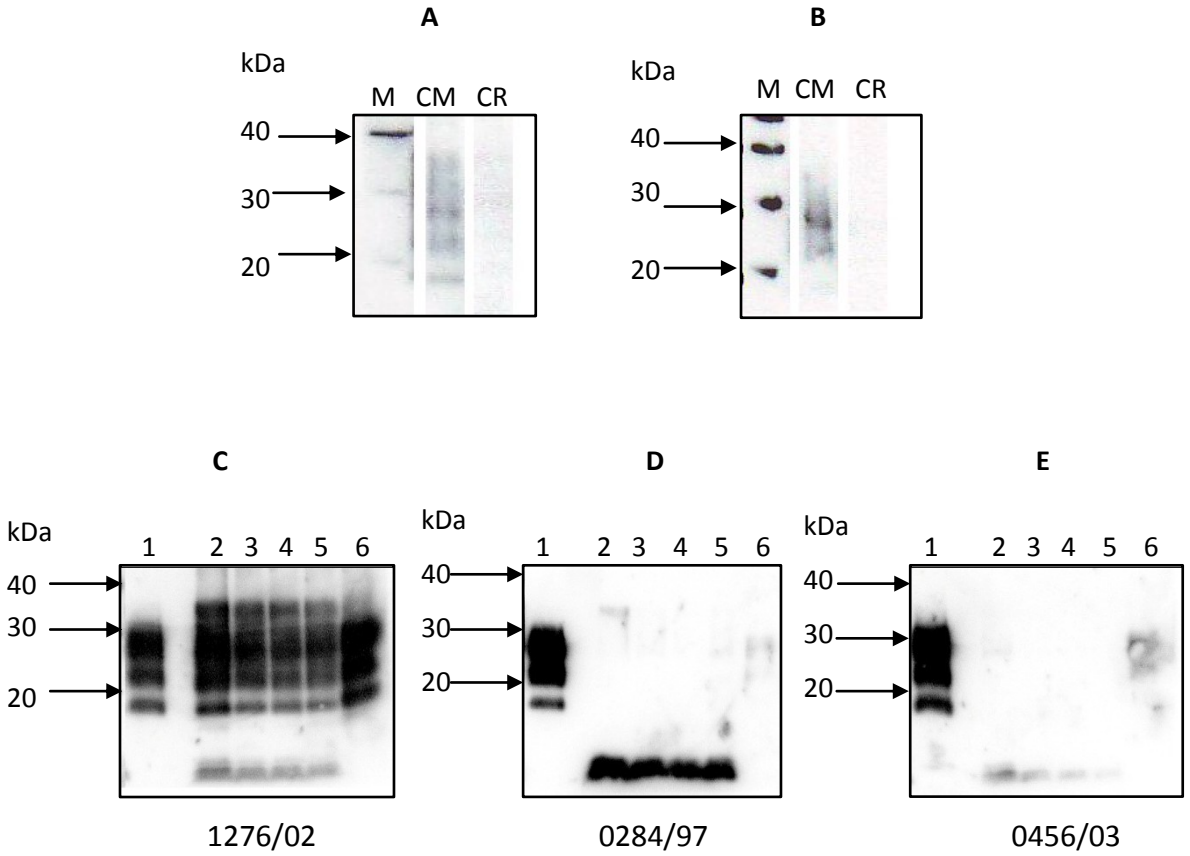


Figure 4.7. Digestion of 10% (w/v) brain homogenates from ovine isolates 0284/97, 0456/03 and 1276/02 with thermolysin or proteinase K. Caudal medulla (CM) and cerebellum (CR) were digested for 8 hours with 150µg/ml/hour thermolysin at 70°C from scrapie infected sheep 0284/97 (panel A) and 0456/03 (panel B). Samples corresponding to 9.9µl of 10% (w/v) digested brain homogenate were loaded per lane and detected using anti-PrP monoclonal antibody L42 diluted 1:10,000. Panels C-E: Western blots of caudal medulla (lane 1) and cerebellum (lanes 2-6) digested with 50µg/ml PK for 1 hour (lanes 1 and 6) or 150µg/ml thermolysin per hour for 8 hours from animals 1276/02 (panel C), 0284/97 (panel D), and 0456/03 (panel E) detected with anti-PrP antibody P4 diluted 1:10,000. For thermolysin digested samples on panels C to E the resulting PrP profiles after 1, 4, 6, and 8 hours are shown in lanes 2 to 5. Samples were visualised using HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

4.2.4 Densitometry analysis of thermolysin-resistant PrP^{Sc} species within the CNS of TSE infected ruminants

Densitometry analysis of Western blot signals following 8 hours thermolysin digestion of caudal medulla 10% (w/v) homogenates was used to quantify the differences described in section 4.2.2. Following exposure of the autoradiograph film to the chemiluminescent signal, an exposure was chosen for digital scanning below the saturation limit of the film, and densitometry analysis performed using Biosoft Quantiscan software. Alternately, chemiluminescent signal was captured using a Photek ICCD225 camera system and images converted to a suitable format for analysis using the Quantiscan software.

In the case of ovine scrapie samples the most prevalent band was at approximately 28kDa, with further bands at 36kDa, 23kDa and 19kDa. For ovine BSE samples no 23kDa or 19kDa bands were produced, and the 36kDa band was at equal or higher intensity to the 28kDa band. Typical results are shown in figures 4.8 and 4.9, and these criteria were used to distinguish between ovine scrapie and ovine BSE cases. Also included for comparison is the typical profile obtained for ovine scrapie strain SSBP/1 following thermolysin digestion of the cerebellum tissue which was available for this experimentally infected animal (figure 4.10).

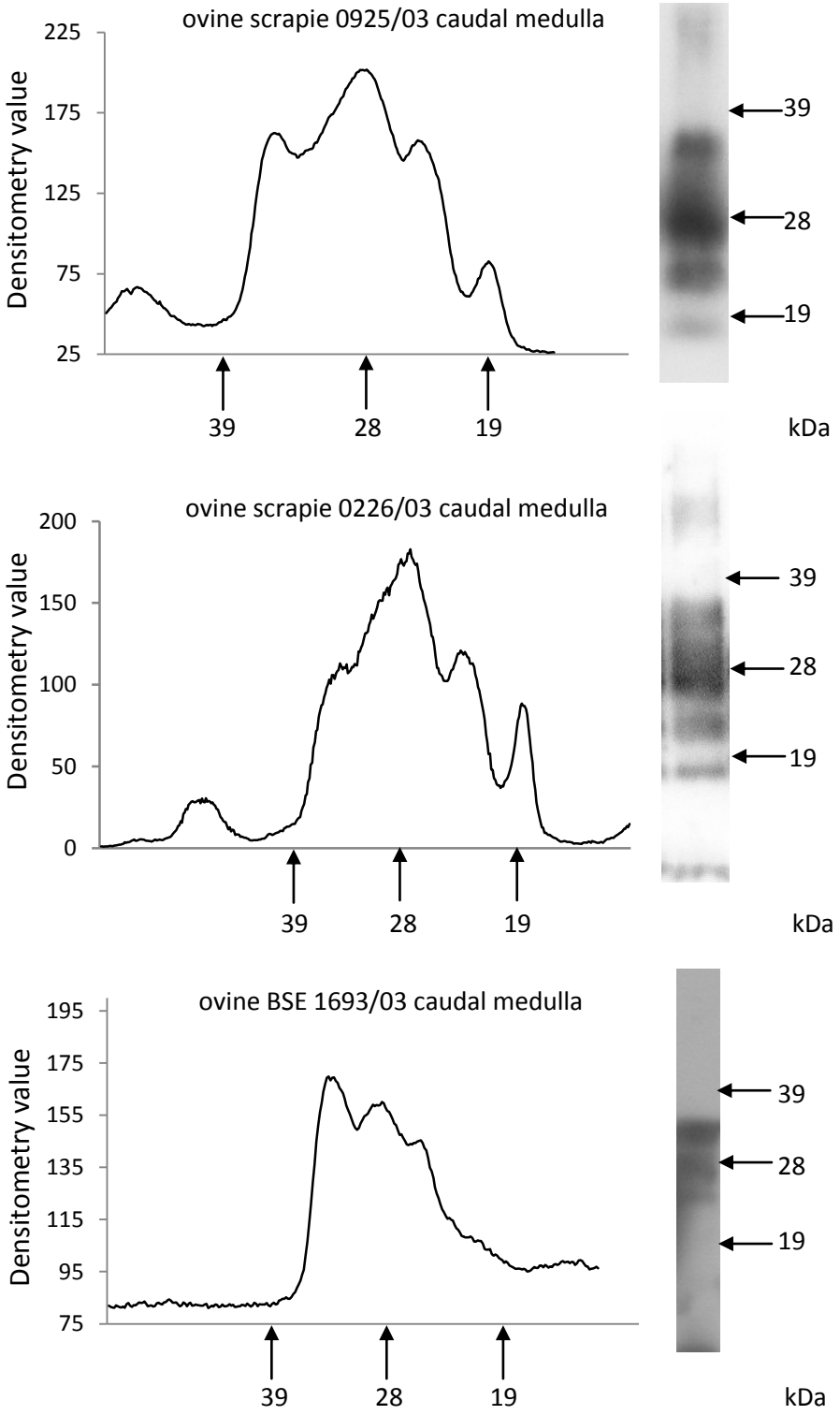


Figure 4.8 Densitometry of thermolysin-resistant PrP^{Sc} profiles generated by Western blotting and P4 (diluted 1:1000) detection of ovine scrapie 0925/03 and 0226/03 caudal medulla and ovine BSE 1693/03 caudal medulla 10% (w/v) homogenates following digestion at 70°C for 8 hours by the addition of 150µg/ml thermolysin per hour.

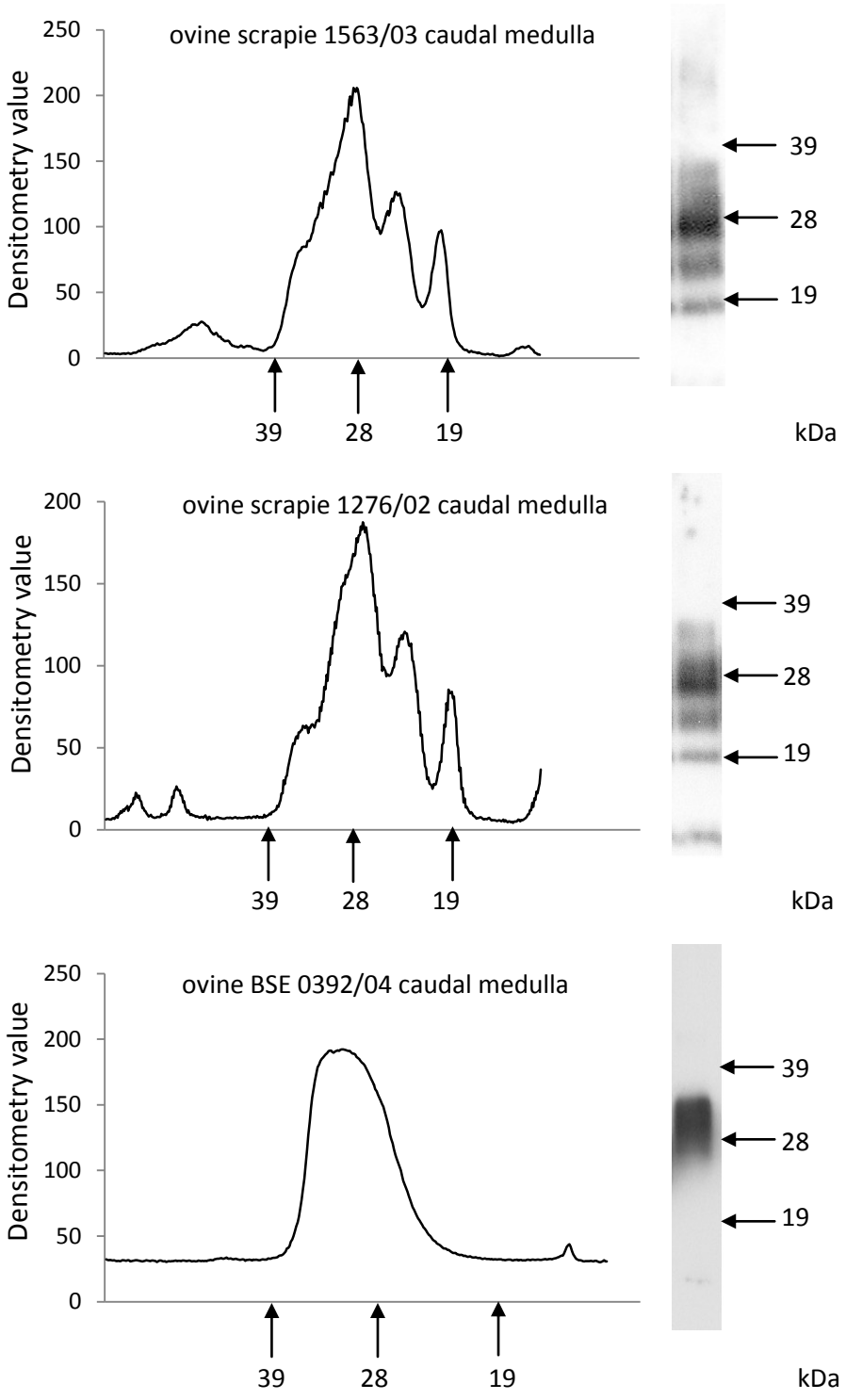


Figure 4.9 Densitometry of thermolysin-resistant PrP^{Sc} profiles generated by Western blotting and P4 (diluted 1:1000) detection of ovine scrapie 1563/03 and 1276/02 caudal medulla and ovine BSE 0392/04 caudal medulla 10% (w/v) homogenates following digestion at 70°C for 8 hours by the addition of 150µg/ml thermolysin per hour.

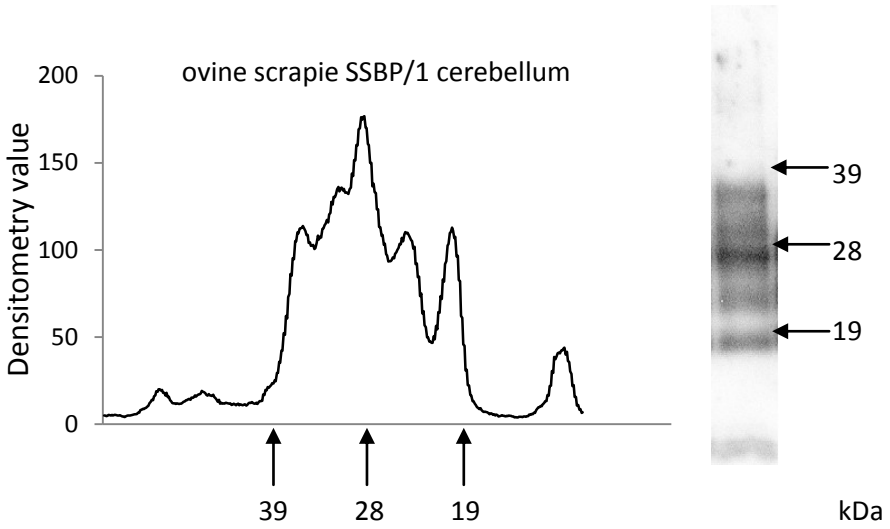


Figure 4.10 Densitometry of thermolysin-resistant PrP^{Sc} profile generated by Western blotting and P4 (diluted 1:1000) detection of experimental ovine scrapie strain SSBP/1 cerebellum 10% (w/v) homogenate following digestion at 70°C for 8 hours by the addition of 150µg/ml thermolysin per hour.

4.2.5 Thermolysin digestion of cerebellum homogenates from sheep experimentally infected with scrapie strains CH1641 and SSBP/1

Western blot analysis of cerebellum homogenates (the only tissue which was available) from sheep experimentally infected with scrapie strains CH1641 (AHQ/AHQ genotype, infected by intracerebral inoculation) and SSBP/1 (VRQ/VRQ genotype, infected by subcutaneous inoculation) using the thermolysin assay indicated considerable differences in the deposition of thermolysin-resistant PrP^{Sc}. Western blot profiles following probing of the blots with anti-PrP monoclonal antibody P4 indicated a scrapie-associated banding pattern with SSBP/1 which had been previously observed with cerebellum homogenates from 12 of 14 natural scrapie cases analysed previously (section 4.2.3), with detectable prion-specific bands at 36, 28, 23, and 19kDa over the 8 hour digestion time course (figure 4.11). PK digestion also confirmed the presence of PK-resistant PrP species typically associated with a TSE-positive status (figure 4.12).

For CH1641, however, no scrapie-specific PrP^{Sc} bands were detected after a single hour digestion with 150µg/ml/hour thermolysin (figure 4.11), displaying a thermolysin-sensitive PrP^{Sc} profile previously observed in cerebellum homogenates from animals 0284/97 (genotype AHQ/AHQ) and 0456/03 (genotype ARQ/VRQ). Whilst the route of infection for these experimental scrapie strains was not identical, it is believed unlikely that this would account for the differences in distribution of thermolysin-resistant PrP^{Sc} that were observed.

The absence of thermolysin-resistant PrP^{Sc} in cerebellum from a CH1641 infected animal was in marked contrast to the presence of PK-resistant PrP^{Sc} in the same sample, as confirmed by Western blotting (figure 4.12), and therefore confirming the

presence of TSE-associated PrP within the CNS of this animal under digestion conditions which resulted in the complete clearance of PrP^{C} from healthy ovine cerebellum homogenate. The presence of PK-resistant PrP^{Sc} in CH1641 cerebellum was notably different to the absence of PK-resistant PrP^{Sc} species from this brain region in scrapie infected sheep 0284/97 and 0456/03 (figure 4.7).

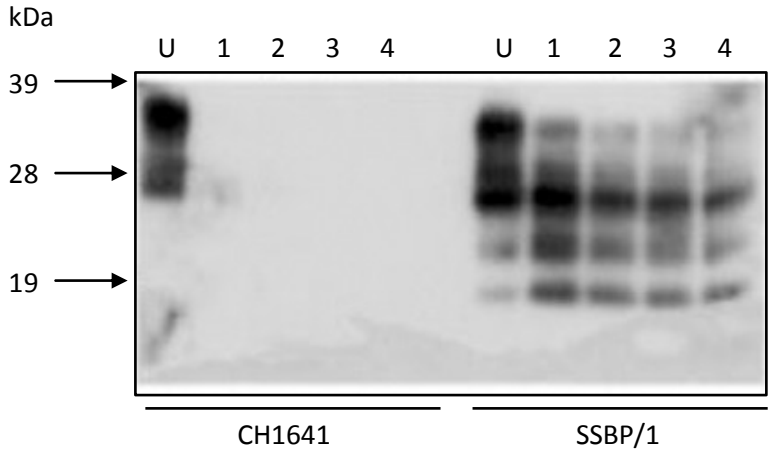


Figure 4.11 Digestion of 10% (w/v) cerebellum homogenate from experimental scrapie strains CH1641 and SSBP/1 with 150µg/ml/hour thermolysin for 8 hours. 3.3µl of 2% (w/v) undigested samples (U) and 3.3µl of 10% (w/v) samples digested for 1, 4, 6, and 8 hours (lanes 1-4) were detected with anti-PrP antibody P4 diluted 1:10,000 and visualised with anti-mouse HRP conjugated antibody and HRP chemiluminescent substrate.

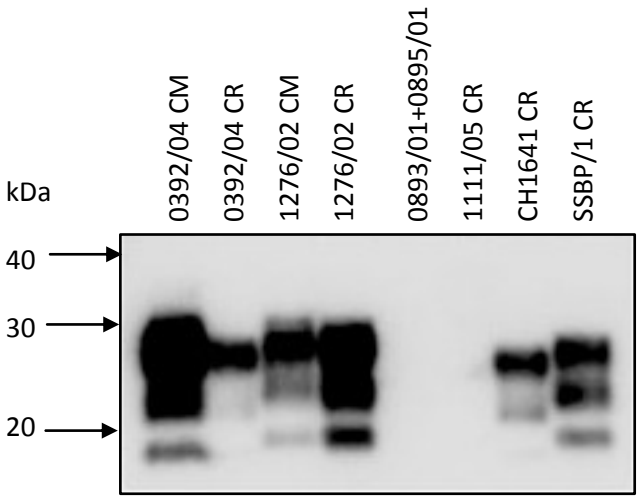


Figure 4.12 Detection of PK-resistant PrP^{Sc} in BSE- and scrapie-infected sheep caudal medulla and cerebellum using antibody P4. Caudal medulla (CM) or cerebellum (CR) 10% (w/v) homogenates from BSE-infected (0392/04), scrapie infected (1276/02, CH1641, and SSBP/1), and healthy sheep (1111/05, and a pool from 2 animals: 0893/01 and 0895/01) were digested for 1 hour with 50µg/ml PK and 3.3µl of 10% (w/v) digested sample loaded per lane. PrP^{Sc} was detected with anti-PrP antibody P4 diluted 1:10,000. Proteins were visualised with anti-mouse HRP conjugated antibody and HRP chemiluminescent substrate.

4.3 Endogenous PrP cleavage within the CNSs of TSE-affected sheep

Previous studies have demonstrated the presence of N-terminally truncated PrP species in scrapie-infected mice (Pan *et al.*, 2005) and humans affected with CJD (Chen *et al.*, 1995), and have reported these PrP species to be glycosylated and non-glycosylated versions of a single PrP fragment. Designated the "C2" species, C2 has not previously been reported to have been observed in scrapie-affected sheep, and therefore an investigation was made into whether C2-like PrP species were also present in the CNS material from natural scrapie hosts. To this end caudal medulla, cerebellum, and spinal cord from 11 scrapie infected sheep of various genotypes were investigated for the presence of C2 PrP fragments (figure 4.13) using antibody P4 (the epitope of which is predicted to be located towards the extreme N-terminus of C2). In 7 of the field cases C2 fragments of approximately 23kDa and 19kDa were detected in all CNS samples. 3 of 11 animals (0456/03, 0455/03, and 0226/03) demonstrated the presence of C2 fragments in the caudal medulla and spinal cord, but not in the cerebellum. A single animal (0284/97) was found not to produce any C2 fragments in any of the three CNS regions tested. Of note was the absence of C2 within the cerebellum from animals 0456/03 and 0284/97, which have previously been demonstrated to contain no PrP^{Sc} within this brain region (section 4.2).

Furthermore, no C2 fragments were observed from either caudal medulla or cerebellum from three BSE-infected sheep (figure 4.11, animals 0392/04, 1693/03, and 0654/03) or from healthy animals of various genotypes in the spinal cord (n=11), cerebellum (n=3) or caudal medulla (n=14) regions (data not shown and figure 4.13). An example of the results typically obtained are displayed in figure 4.14, and indicated

an absence in C2 PrP fragments in spinal cord, cerebellum, or caudal medulla from 6 TSE-negative sheep, in contrast to the presence of 23kDa and 19kDa C2 PrP fragments in spinal cord tissue from 6 scrapie-positive animals.

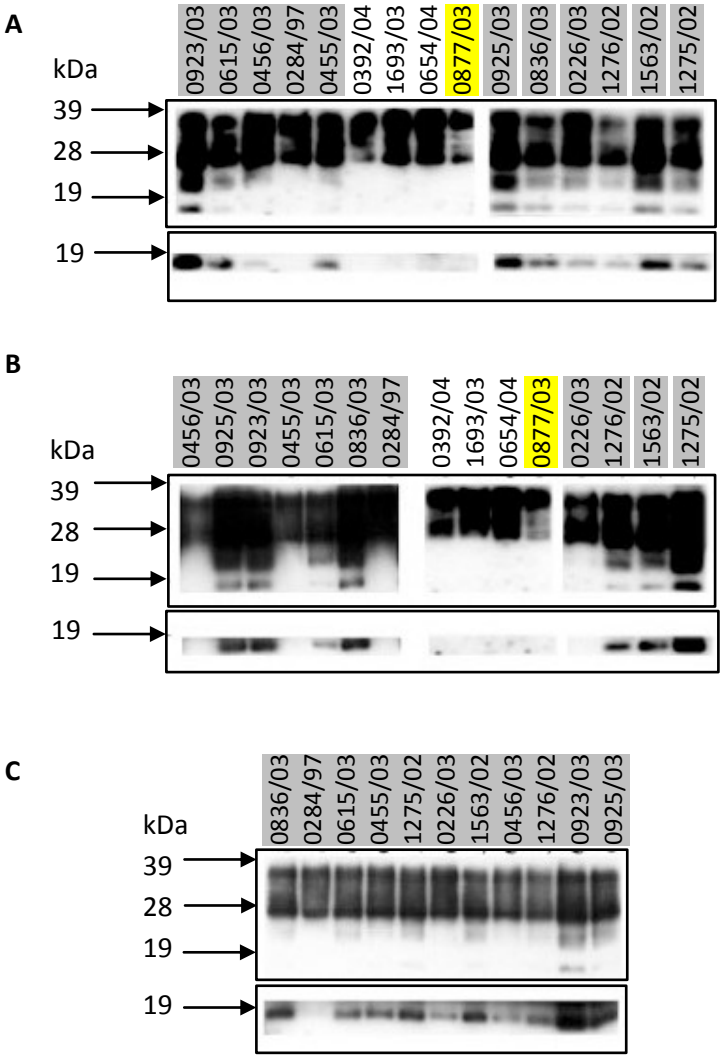


Figure 4.13 Distribution of C2 PrP species within the CNS of TSE infected sheep. Typically 3.3µl of 2% (w/v) undigested homogenate prepared from caudal medulla (panel A), cerebellum (panel B) or spinal cord (panel C) was loaded per lane from scrapie infected sheep (grey highlight), experimental BSE infected sheep (no highlight), or healthy sheep (yellow highlight) as indicated. Over-exposure of the Western blots to highlight the C2 fragment are shown in the lower panels. PrP was detected with antibody P4 diluted 1:10,000 (panels A and B) or 1:5,000 (panel C) and anti-mouse HRP conjugated antibody. Signals were visualised using HRP chemiluminescent substrate.

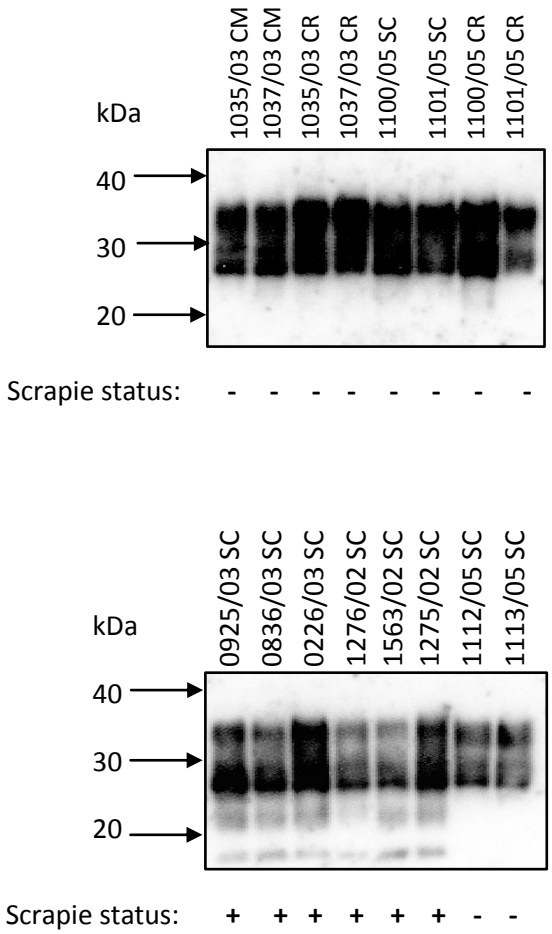


Figure 4.14 Detection of C2 PrP fragments in caudal medulla, cerebellum and spinal cord homogenates from healthy sheep and naturally-occurring scrapie sheep cases. 2% (w/v) homogenates were loaded to approximately equal PrP signals and PrP detected using anti-PrP antibody P4 diluted 1:10,000. All positive samples had 3.3µl of 2% (w/v) homogenate applied per lane with the exception of spinal cord from animal 0226/03 which had 5µl 2% (w/v) homogenate loaded. Negative sample loadings ranged from 3.3µl to 6.6µl of 2% (w/v) homogenate. With the exception of spinal cord from scrapie-infected animal 0226/03, healthy-ovine sample loadings either equalled or exceeded sample loadings from scrapie positive animals.

Analysis of cerebellum homogenates by Western blot from sheep experimentally infected with scrapie strains CH1641 and SSBP/1 indicated that while truncated PrP fragments were endogenously produced in an SSBP/1 infected animal, no C2 fragments could be detected from the CH1641 infected animal (figure 4.15, top panel). Identification of the cleavage site resulting in the formation of C2 PrP fragments was performed by probing Western blots with either anti-PrP monoclonal antibodies P4 and SAF32. The absence of detectable 23kDa and 19kDa C2 fragments when probed with SAF-32, in contrast to the detection of C2 fragments with antibody P4, suggested that the cleavage site lies between the SAF32 and P4 epitopes, and that both the 23kDa and 19kDa C2 fragments contain similar N-terminal cleavage sites (figure 4.15).

The apparent strain specific production of C2 fragments is believed to occur as a result of endogenous processing of the PrP protein as opposed to proteolytic cleavage arising as an artefact during preparation of the homogenates, since incorporation of protease inhibitors and EDTA into the lysis buffer used to prepare homogenates had no effect on C2 fragment production (figure 4.16, panel A)

Deglycoslylation of caudal medulla homogenate from scrapie infected animals 0455/03 and 0456/03 using PNGase F resulted in the deglycosylation of mono-and di-glycosylated PrP, and the corresponding production of a single deglycosylated protein which migrated at approximately 27kDa, i.e. at approximately the size corresponding to full length unglycosylated PrP (figure 4.16, panel B). C2 fragments of approximately 23kDa and 19kDa resolved to a single 19kDa C2 fragment following deglycosylation (figure 4.16, panel B).

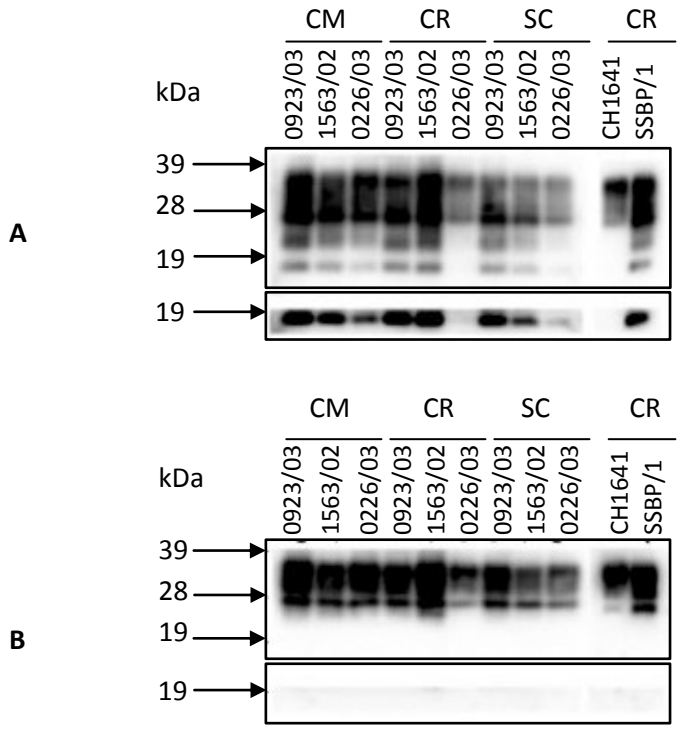


Figure 4.15. Analysis of field scrapie cases and experimental ovine scrapie strains with antibodies P4 and SAF32 targeted to the N-terminal region of PrP. Samples consisting of 3.3µl of 2% (w/v) caudal medulla (CM), cerebellum (CR), or spinal cord (SC) from natural scrapie cases 0923/03, 1562/02, and 0226/03, or experimental ovine scrapie strains CH1641 and SSBP/1 were separated on 12% (w/v) NuPAGE Novex gels. PrP was detected using anti-PrP antibodies P4 (panel A, and over-exposed lower panel A) diluted 1:5,000 or SAF32 (panel B, and over-exposed lower panel B) diluted 1:20,000 (field cases) or 1:10,000 (CH1641 and SSBP/1) in PBST. PrP was visualised using HRP chemiluminescent substrate following detection of monoclonal antibodies P4 and SAF32 with HRP conjugated anti-mouse antibody.

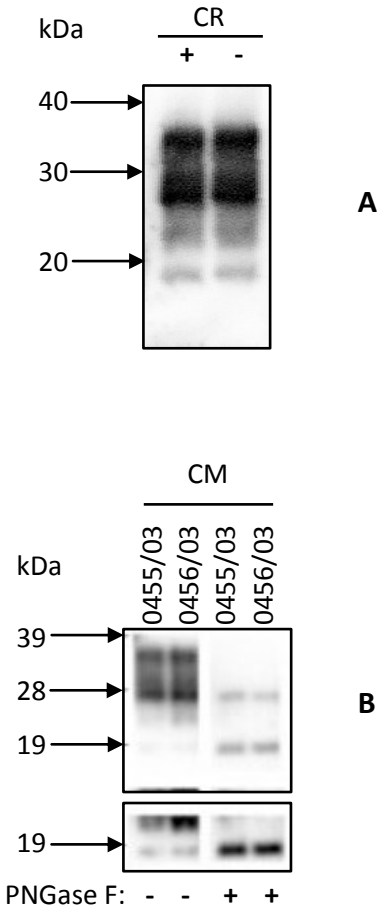


Figure 4.16. Analysis of the effect of protease inhibitors and EDTA on the generation of C2 fragments during CNS homogenate preparation, and the deglycosylation of C2 fragments using PNGaseF. Top panel: 3.3µl of 2% (w/v) cerebellum homogenate (CR) from field scrapie animal 0836/03 was prepared using standard methods in brain homogenate lysis buffer alone (-) or lysis buffer containing 1X Roche Complete Protease Inhibitor and 1mM EDTA (+). PrP was detected using anti-PrP antibody P4 diluted 1:5,000. Lower panels: Deglycosylation of caudal medulla homogenate from scrapie animals 0455/03 and 0456/03 with PNGase F. Samples were subjected to PNGase F treatment as described in section 2.2.3 (+) or untreated (-) and separated on 12% (w/v) Invitrogen NuPAGE Novex gels prior to detection of PrP with anti-PrP antibody P4 diluted 1:5,000. Signals were visualised with HRP conjugated anti-mouse antibody and HRP chemiluminescent substrate.

A summary of results from the analysis of 11 field scrapie cases for which spinal cord, caudal medulla, and cerebellum were available, in addition to cerebellum from ovine scrapie strains CH1641, SSBP/1, and a further 4 field ovine scrapie animals, and 4 experimentally infected ovine BSE animals for which a more limited range of tissues were available, is given in table 4.1.

Animal	Genotype	Breed (age in yr)	PrP^{Sc} type within CM	PrP^{Sc} within		C2 within		
				SC	CR	CM	SC	CR
0455/03	VRQ/VRQ	Swaledale (6)	Scrapie	+	+	+	+	-
0226/03	ARQ/VRQ	Swaledale (5)	Scrapie	+	+	+	+	-
0456/03	ARQ/VRQ	Swaledale (3)	Scrapie	+	-	+	+	-
0284/97	AHQ/AHQ	Finn Dorset (unknown)	Scrapie	+	-	-	-	-
0615/03	ARQ/ARQ	Swaledale (5)	Scrapie	+	+	+	+	+
0923/03	VRQ/VRQ	Welsh Hill Speckled (2)	Scrapie	+	+	+	+	+
0925/03	VRQ/VRQ	Welsh Hill Speckled (5)	Scrapie	+	+	+	+	+
0836/03	VRQ/VRQ	Mule (2)	Scrapie	+	+	+	+	+
1276/02	VRQ/VRQ	Welsh Mountain (2)	Scrapie	+	+	+	+	+
1563/02	VRQ/VRQ	Bleu De Maine (3)	Scrapie	+	+	+	+	+
1275/02	VRQ/VRQ	Welsh Mountain (2)	Scrapie	+	+	+	+	+
0210/03	ARQ/ARQ	Warborough (2)	Scrapie	ND	+	ND	ND	ND
0635/03	ARQ/ARQ	Charollais Cross (3)	Scrapie	ND	+	ND	ND	ND
0678/03	ARQ/ARQ	Suffolk Cross (3)	Scrapie	ND	+	ND	ND	ND
0575/00	ARQ/ARQ	Cambridge (3)	Scrapie	ND	ND	ND	ND	ND
0392/04	ARQ/ARQ	Romney (3)	BSE	ND	+	-	ND	-
1693/03	ARQ/ARQ	Romney (2)	BSE	ND	+	-	ND	-
0654/04	ARQ/ARQ	Romney (3)	BSE	ND	+	-	ND	-
0394/04	ARQ/ARQ	Romney (unknown)	BSE	ND	ND	ND	ND	ND
CH1641	AHQ/AHQ	Cheviot (1)	ND	ND	-	ND	ND	-
SSBP/1	AHQ/AHQ	Cheviot (2)	ND	ND	+	ND	ND	+

Table 4.1. Analysis of CNS tissue homogenates tested using the thermolysin strain-typing assay to determine prion strain, and scoring of caudal medulla (CM), cerebellum (CR), and spinal cord (SC) for the presence (+) or absence (-) of C2 prion fragments. ND is not determined. Animals tested were natural infections of scrapie with the exception of CH1641, SSBP/1 and 4 experimentally infected ovine BSE cases.

CHAPTER 5

The persistence of ruminant TSEs in UK soils

5.1 Introduction

This chapter describes the elution and persistence of two TSE strains (ovine scrapie and bovine BSE) on a range of UK soils under experimental conditions over an 18 month period. Factors such as the migration of PrP^{Sc} within soil columns and the distribution of PrP^{Sc} to soil particles fractionated by size were also examined. A single soil displaying high persistence and elution of PrP^{Sc} was further investigated into the effect of factors such as defined pH, temperature, and moisture content of soil on the elution, persistence and migration of PrP^{Sc}.

5.2.1 Soil characteristics

6 UK soils were selected from ADAS UK Ltd. farm sites representing a range of UK lowland agricultural soil types for which there was no recorded history of farm animal occupation. Sites of soil collection were located in Cambridgeshire (n=3), Nottinghamshire (n=2), and Herefordshire. OSGB grid references for all 6 soils are given in table 2.4.

Soils were characterised in order determine their respective composition and mineralogy so that the behaviour of PrP^{Sc} could be better understood in relation to the soil characteristics. Determinations of soil biomass and soil respiration rates were performed by ADAS Ltd (Boxworth, UK). All other analyses were performed by Eurofins Ltd (Wolverhampton, UK). Mineral characteristics were based on the inorganic fraction only (table 5.1).

Classification of the 6 UK soils within a ternary plot according to the United States Department of Agriculture (USDA) soil textural classification demonstrated the wide range of textures represented by the soils (figure 5.1, produced by George Shaw, University of Nottingham). Based on the soil textural characteristics (figure 5.1) the six soils were classified as follows: Notts1 – sandy loam, Notts2 – loamy sand, Cambs1 – clay loam/clay, Cambs2 – clay, Cambs3 – clay, and Hereford – silt loam/silty clay loam.

	Notts1	Notts2	Cambs1	Cambs2	Cambs3	Hereford
pH	6.2	6.6	6.8	7.5	5.8	6.3
organic matter %	3	2	8	39	3	3
moisture content %[a]	13	9	26	63	17	25
Porosity %[b]	45	44	45	62	55	32
sand (>63µm) %	62	86	33	8	27	10
silt (63-2µm) %	27	7	27	35	29	63
clay (<2µm) %	11	7	40	57	44	27
Soil Respiration - mg CO_2-C per kg per day	2.3	2.2	14.1	21.2	2.4	10.1
Al (g/kg)[c]	8.73	4.75	27.10	25.20	30.70	33.40
Al_2O_3 (%)[c]	3.3	1.8	10.2	9.5	11.6	12.6
Fe (g/kg) [c]	13.50	8.31	33.30	32.00	33.40	36.10
Fe_2O_3 (%)[c]	3.9	2.4	9.5	9.2	9.6	10.3
Mn (g/kg)[c]	0.56	0.24	0.27	0.53	0.56	1.04
MnO_2 (%)[c]	0.09	0.04	0.04	0.08	0.09	0.16

[a] calculated as the percentage water per dry weight of soil

[b] the effective porosity of the soils based on the bulk densities reported for the columns

[c] calculated in relation to dry mass of soil

Table 5.1 Characteristics of 6 UK soils selected for analysis of PrP persistence. Soil characteristics were determined by Eurofins Ltd using standard methods defined in M.A.F.F publication RB427 “The Analysis of Agricultural Materials” published in 1986 by HMSO (London, UK). Elemental aluminium, iron, and manganese concentrations were determined using Inductively Coupled Plasma-Optical Emission Spectroscopy by Eurofins Ltd. Determination of soil respiration rate was performed by ADAS UK.

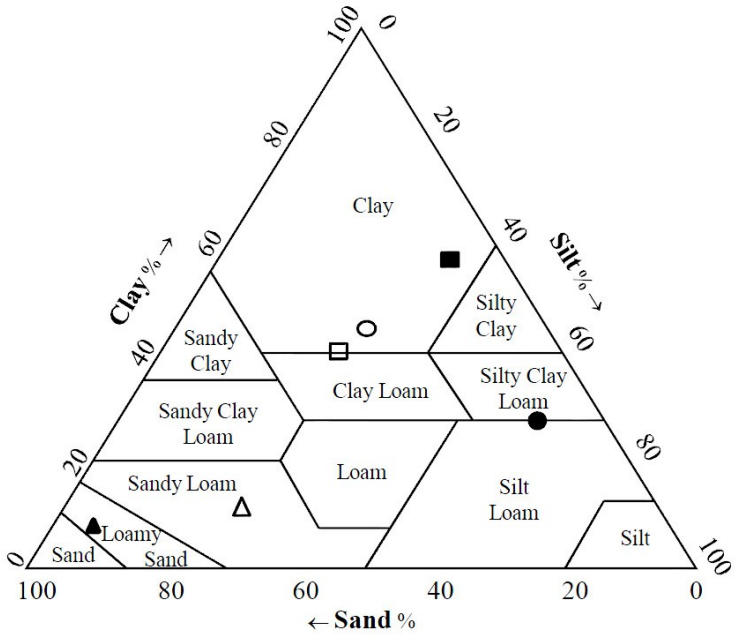


Figure 5.1. Soil ternary plot produced using the USDA classification of 6 UK soils selected for the analysis of ruminant PrP^{Sc} persistence (generated by George Shaw, University of Nottingham). Soils were Notts1 (△), Notts 2 (▲), Cambs 1 (□), Cambs2 (■), Cambs3 (○), and Hereford (●).

5.2.2 Binding of PrP to soil and elution of PrP^{Sc} from soil using proteinase K and SDS

Initial experiments were performed to determine a suitable prion elution methodology from soil based on that described by Johnson and co-workers, using SDS for the extraction of PrP^{Sc} from 6 UK soils (Johnson *et al.*, 2007) . Soils spiked with 10µl 20% (w/v) brain homogenate per 100mg soil were incubated for 24 hours at 16-20°C and 0.5g aliquots of soil extracted as described previously (section 2.2.13). Analysis of extracts by Western blotting indicated PrP^{Sc} bound to all soil types and could be desorbed from all soils using a combination of protease digestion and boiling in SDS (figure 5.2, panel A), though considerable variability in extraction efficiency was observed between the soils. Maximum recovery of PrP^{Sc} was observed with Notts1 and Notts2 soils (>20% recovery), with intermediate recovery (between 5% and 20%) observed with Hereford soil. Extraction of PrP^{Sc} from organic and clay rich soils Cambs1, Cambs2, and Cambs3 resulted in 5% or less recovery of spiked PrP^{Sc} after 24 hours, indicating the rapid and irreversible binding of the majority of PrP^{Sc} to these soils. Elution was particularly inefficient from Cambs2 soil which contained a relatively high fraction of organic material.

Extraction of ovine and bovine PrP^{C} from those soils which gave the most efficient PrP^{Sc} extraction, i.e. Notts1 and Notts2, following 24 hours incubation with 15µl of 20% (w/v) healthy ovine or healthy bovine brain homogenate per 100mg soil (i.e. 150% of the standard spike of 10µl 20% (w/v) brain homogenate used when preparing the soil columns) indicated that no recoverable PrP^{C} was present following digestion with 50µg/ml PK for 1 hour at 37°C and SDS extraction. This indicated that PrP signals obtained by Western blotting following SDS elution of PrP^{Sc} from soil samples were due to eluted PrP^{Sc} and not eluted, undigested PrP^{C} (figure 5.2, panel B). Furthermore,

no notable difference was observed in the extraction efficiency between ovine PrP^{Sc} or bovine PrP^{Sc} on Notts1 or -2 soils (figure 5.2, panel B).

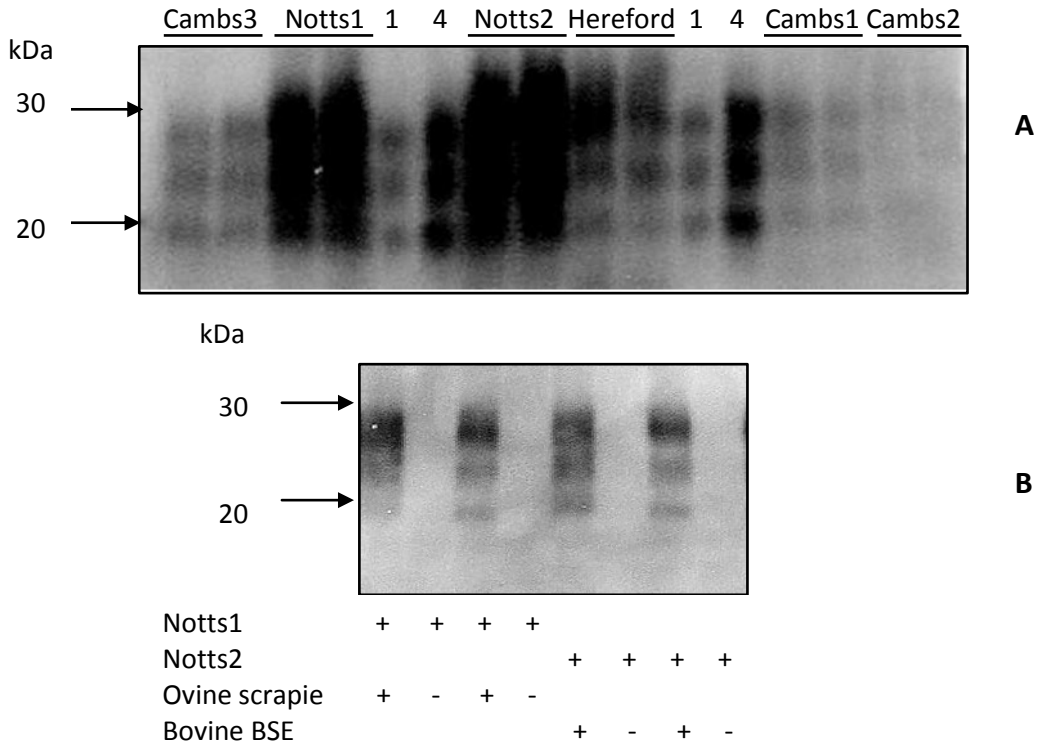


Figure 5.2. Detection of PrP^{Sc} by Western blotting following elution of soil bound PrP^{Sc} using 50ug/ml PK and 10% (w/v) SDS. Duplicate samples (equivalent to 100mg soil) were extracted following 24 hours binding of 10µl 20% (w/v) ovine scrapie brain homogenate per 100mg soil at 16-20°C on each soil as indicated in panel A. Direct loadings of 1µl (1) and 4µl (4) of 10% (w/v) ovine scrapie brain homogenate digested with 50µg/ml PK were used to determine approximate recoveries following detection using anti-PrP antibody SHA31 and alkaline phosphatase conjugated anti-mouse antibody.

Panel B: Western blot of the elution of ovine and bovine PrP^{Sc} and PrP^{C} following incubation of healthy ovine (-), scrapie ovine (+), healthy bovine (-) and BSE infected bovine (+) brain homogenates with Notts1 and Notts2 soils at 15µl 20%(w/v) brain homogenate per 100mg soil for 24 hours (bottom panel) and eluted with 50µg/ml PK and SDS. 200mg equivalent soil extracts were loaded per lane as indicated and detected with anti-PrP antibody SHA31 and AP conjugated anti-mouse antibody.

5.2.3 Effect on the efficiency of methanol precipitation of PrP^{Sc} by soil supernatant

In order to confirm that unbound PrP^{Sc} could be recovered from soil supernatant an assessment of the effect of soil supernatant was made on the efficiency of methanol precipitation of PrP^{Sc}. A comparison of methanol precipitation efficiency between PrP^{Sc} spiked into 500µl 5mM calcium chloride and the equivalent volume of soil supernatant was performed. Soil supernatant was prepared by resuspending 100mg of Notts1 or Notts2 soil in 500µl 5mM calcium chloride followed by centrifugation at 800xg for 10 minutes. Supernatant and calcium chloride solutions were spiked with 10µl 20% (w/v) of brain homogenate digested with 50µg/ml PK for 1 hour and PrP^{Sc} recovered by centrifugation at 12100xg for 30 minutes following methanol precipitation with 5 volumes of methanol. No difference in the recovery of PrP^{Sc} was detected between soil supernatant and calcium chloride solution, suggesting there was no discernable inhibition of precipitation by factors present in those two soils (figure 5.3).

Furthermore, following 24 hours co-incubation of Notts1 and Notts2 soils with healthy ovine or healthy bovine brain homogenates in the absence of protease digestion indicated only low levels of PrP^{C} were recovered by methanol precipitation of soil supernatants prepared by centrifugation of the soil/homogenate mixtures at 800xg for 10 minutes (figure 5.3). This indicated rapid and efficient binding of PrP^{C} to the two Notts soils which have previously been demonstrated to offer the highest levels of PrP^{Sc} recovery.

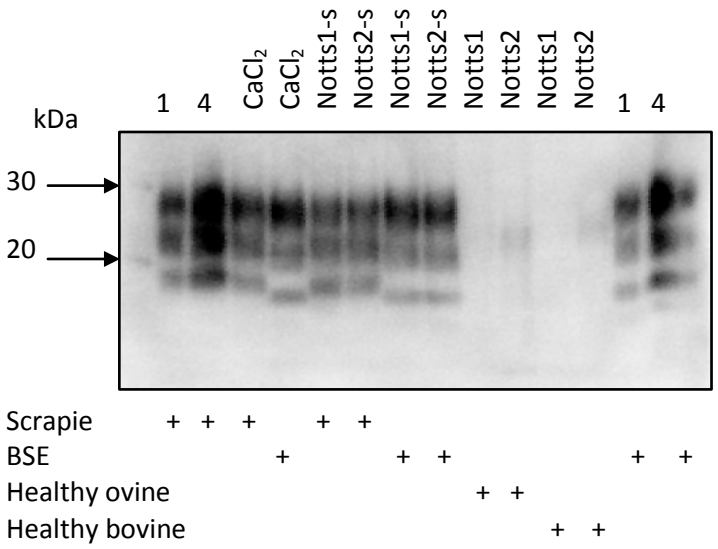


Figure 5.3. Methanol precipitation efficiency of 10µl 10% (w/v) PK digested scrapie infected ovine and BSE infected bovine brain homogenates spiked into either 500µl 5mM calcium chloride or 500µl Notts1 or Notts2 soil supernatant. Soil supernatants (Notts1-s, Notts2-s) were generated by the addition of 500µl 5mM calcium chloride to 100mg of each soil and pelleted at 800xg for 10 minutes. Spiked buffer or spiked soil supernatants were precipitated with 5 volumes of methanol for 16 hours at -20°C and centrifuged at 12100xg for 30 minutes prior to resuspension in 2x NuPAGE loading buffer. The equivalent of 2µl 10% (w/v) brain homogenate of initial spike was loaded per lane. For recovery of PrP^C from Notts1 and Notts2 soils the supernatant obtained following incubation of 100mg of each soil with 10µl 20% (w/v) healthy brain homogenate at 16-20°C for 24 hours was recovered by methanol precipitation and all the sample loaded. Direct loadings of 1ul 10% (w/v) PK digested TSE infected brain homogenate (1) and 4µl of 10% (w/v) PK digested TSE infected brain homogenate (4) as controls are indicated. PrP was detected on the Western blot with anti-PrP antibody SHA31 diluted 1:2,000 and AP conjugated anti-mouse secondary antibody. Western blots were visualised by the addition of Invitrogen AP Western blotting substrate.

5.2.4 Comparison of elution efficiency between ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} from 6 UK soils following 24 hours co-incubation on soil

As has been previously noted, considerable differences were observed between the elution efficiencies of ovine scrapie PrP^{Sc} on the 6 UK soils when analysed for their capacity to bind PrP^{Sc} over a 24 hour period (figure 5.2, panel A). A comparison of the elution efficiency of bovine BSE PrP^{Sc} from the 6 UK soils following 24 hours co-incubation (10µl 20% (w/v) CNS homogenate per 100mg soil at 16-20°C) indicated similar elution trends to that of scrapie PrP^{Sc} (figure 5.4). This suggested that soil type rather than TSE strain was the largest contributory factor in the elution efficiency of PrP^{Sc} from the 6 UK soils.

Binding of PrP^{Sc} was essentially complete after 24 hours co-incubation on soil, as proteinase K digestion followed by methanol precipitation of the soil supernatants obtained by centrifugation of a 20%(w/v) slurry of soil/PrP^{Sc} prepared after 24 hours binding within a soil column did not yield any recoverable PrP^{Sc} (figure 5.4, panels B and D). Soils which had displayed the lowest efficiency of PrP^{Sc} desorption (e.g. Cambs2 and Cambs3) were not observed to produce a concomitant increase in PrP^{Sc} recovery from the supernatant, suggesting that desorption efficiency from Cambs2 soil with a relatively high organic content was low for both ovine scrapie and bovine BSE PrP^{Sc} and that it was not due to an absence of binding to these soil.

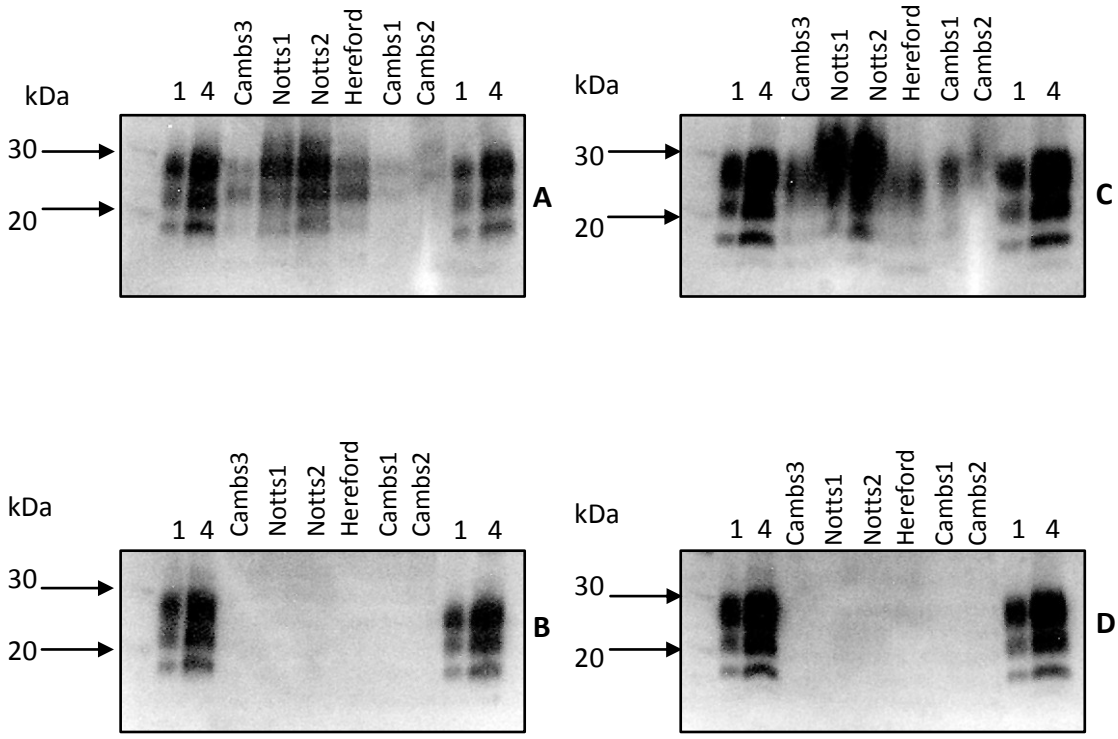


Figure 5.4. Recovery of bound and unbound PrP^{Sc} from 6 UK soils following 24 hours incubation with diseased ovine and diseased bovine CNS homogenates. 0.5g soil samples spiked with 10µl 20% (w/v) ovine scrapie (panels A and B) or bovine (panels C and D) brain homogenates were resuspended in 2.5ml 5mM calcium chloride and centrifuged to provide soil pellets for PK/SDS extraction (panels A and C) and supernatants (panels B and D). Supernatants were digested with 50µg/ml PK prior to methanol precipitation and the resulting pellet loaded onto each lane at a loading of 200mg soil equivalent. For soil extractions the equivalent of 200mg soil extract was loaded per lane from Cambs3, Notts1, Notts2, Hereford, Cambs1, and Cambs2 (as indicated), along with 1µl (1) and 4µl (4) 10% (w/v) PK digested scrapie infected ovine (left panels) and BSE infected bovine (right panels) brain homogenates. Western blots were detected with anti-PrP antibody SHA31 and HRP-conjugated anti-mouse antibody and visualised using HRP chemiluminescent substrate.

5.2.5. N-terminal truncation of PrP^{Sc} upon elution from UK soils

Previous work had demonstrated that upon elution of PrP^{Sc} from certain clay minerals performed in the absence of proteolytic enzymes, truncation of the N-terminal region of PrP^{Sc} had been observed (Johnson *et al*, 2006). Therefore, an investigation was made into whether ovine scrapie PrP^{Sc} was similarly truncated upon elution from the six UK soils under analysis within this work. Elution of ovine scrapie PrP^{Sc} using thermolysin (which has been shown to digest PrP^{C} but leave full length PrP^{Sc} intact) confirmed the findings of Johnson and co-workers.

N-terminal truncation of ovine scrapie PrP^{Sc} was identified by a reduction in chemiluminescent signal when Western blots containing PrP^{Sc} eluted from soil were probed with antibody SAF32 which binds to an epitope in the octapeptide repeat region located within the N-terminal region of PrP. In contrast, detection with antibody SHA31 (targeted against the core region of PrP^{Sc}) demonstrated no comparable reduction in chemiluminescent signal (figure 5.5), and could be used as an indication of the total level of PrP^{Sc} eluted from each particular soil against which levels of N-terminally truncated could be qualitatively compared.

Elution of PrP^{Sc} from the sand-rich Notts1 and Notts2 soils was found to yield full-length PrP^{Sc} at similar levels to total PrP^{Sc} (figure 5.5 and data not shown). In contrast, no full length PrP^{Sc} could be detected from the clay-rich soils Cambs3 (figure 5.5), -1, or -2 (data not shown), despite the presence of truncated PrP^{Sc} being readily detected in eluates from the soils. Full-length PrP^{Sc} was obtained from the silt-rich Hereford soil, albeit at a lower level than total PrP^{Sc}, possibly indicating that the eluate was composed of a mixture of both full-length and N-terminally truncated PrP^{Sc}.

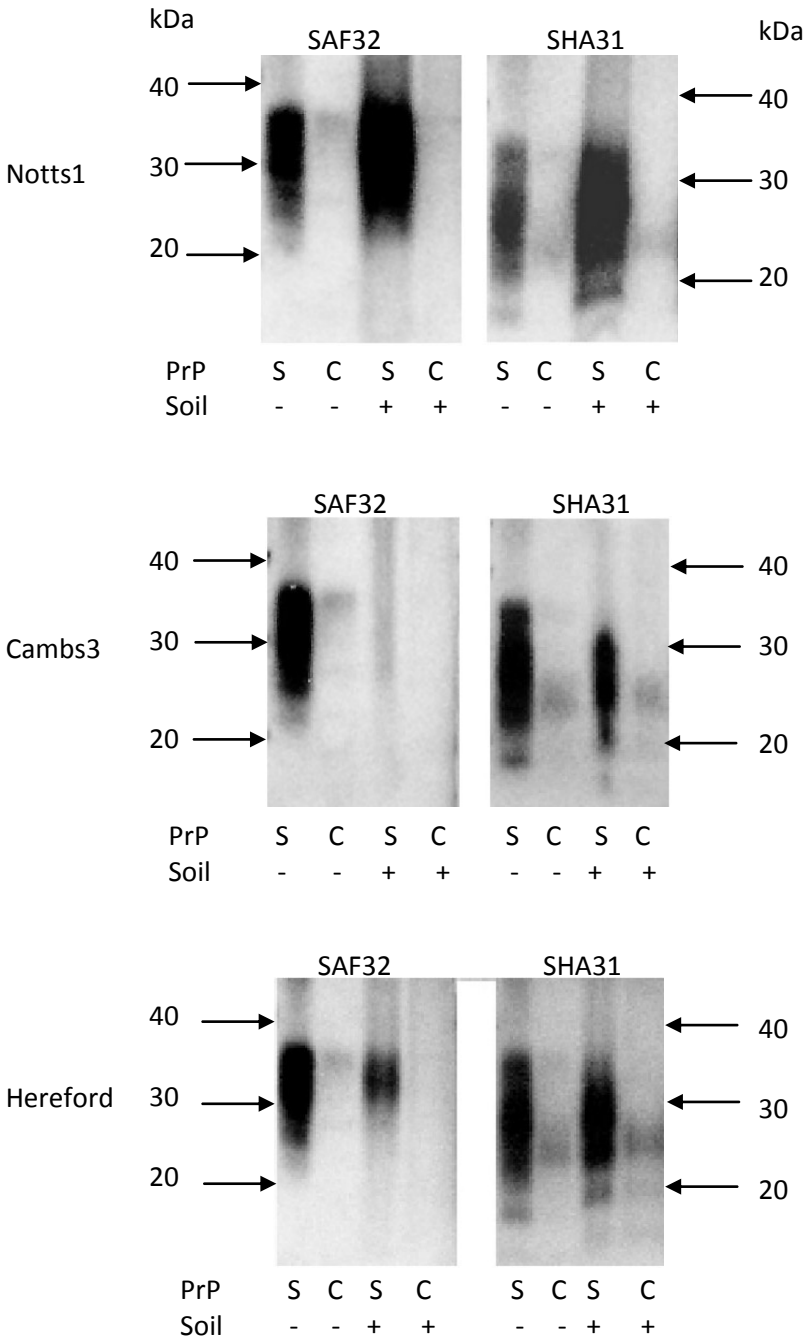


Figure 5.5. N-terminal truncation of ovine scrapie PrP^{Sc} upon elution from 3 UK soils. Brain homogenate from 200mg Notts1, Cambs3, or Hereford soils 24 hours after spiking with 10µl 20% (w/v) ovine scrapie brain homogenate (S) or healthy brain homogenate (C) per 100mg soil was eluted using thermolysin and SDS as described previously (+ soil), and analysed alongside 2µl 10% (w/v) of brain homogenate (- soil) representing a recovery of 5% of the original spike. PrP was detected on Western blot with anti-prion antibodies SAF32 diluted 1:40,000 or SHA31 diluted 1:20,000 as indicated. Blots were visualised with HRP chemiluminescent substrate.

5.2.6. Persistence of ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} in 6 UK soils over an 18 month period

In order to analyse the persistence of ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} on soil over an extended period, Western blot analysis was performed on PrP^{Sc} eluted from six UK soils following co-incubation with 10µl 20%(w/v) CNS homogenates per 100mg soil in columns maintained at 16-20°C for a period of approximately 18 months.

Soil samples representative of the whole column depth were collected after 24 hours, and 1, 3, 6, 9, 12, 15, and 18 months post inoculation. Considerable differences were observed in the persistence of both ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} on the soils over an 18 month period, with recoverable PrP^{Sc} levels for both TSE strains decreasing over time. Persistence could be divided into three groups, namely, relatively high recovery of scrapie ovine PrP^{Sc} from soils Notts1, Notts2, and Cambs3 after 18 months (figure 5.6), intermediate recovery from Hereford soil with ovine scrapie PrP^{Sc} detectable at 9 months post inoculation, and relatively low recovery from the soils rich in organic matter; Cambs1 and Cambs2 (figure 5.7), where ovine scrapie was only readily desorbed after 24 hours and 1 month respectively. A similar pattern was observed with bovine BSE PrP^{Sc}, with the exception that recovery of detectable PrP^{Sc} was reduced in Hereford soil to 3 months and in Cambs3 soil to 6 months (figures 5.6 and 5.7).

To confirm that the reduction in PrP^{Sc} observed was due to interaction with soil an analysis of ovine scrapie brain homogenate maintained at 16-20°C for a period of 6 months in the absence of soil was performed and indicated little reduction in either

total PrP or PrP^{Sc} over the time period, indicating endogenous protease activity was not responsible for the loss of PrP^{Sc} during co-incubation with soil (figure 5.8).

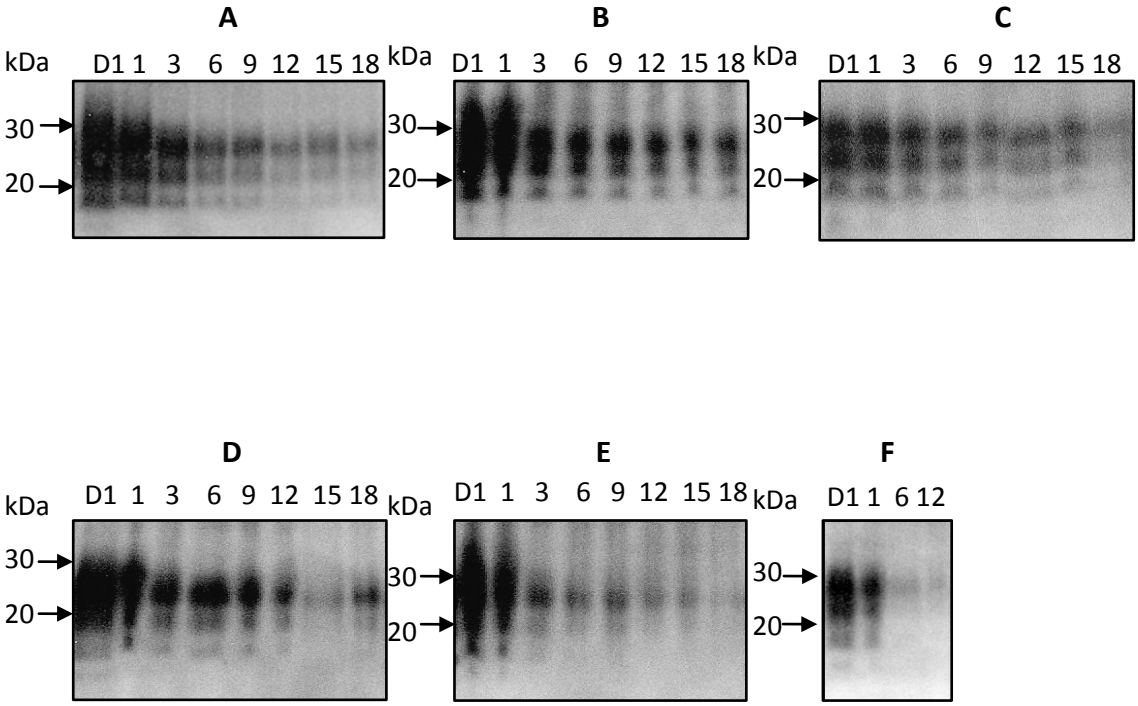


Figure 5.6. Persistence of ovine scrapie and bovine BSE PrP^{Sc} in Notts1, Notts2, and Cambs3 soils over an 18 month period. Soil samples (0.5g) of Notts1 (panels A and D), Notts2 (panels B and E), and Cambs3 (panels C and F) were extracted after 24 hours (D1), 1 month, 3 months, 6 months, 9 months, 12 months, 15 months, and 18 months (as indicated) co-incubation with 10µl 20% (w/v) ovine scrapie (panels A-C) or bovine BSE (panels D-F) brain homogenate per 100mg soil. Following SDS PAGE of 200mg soil equivalent samples, Western blots were probed with anti-PrP antibody SHA31 diluted 1:20,000 and anti-mouse HRP conjugated antibody, and blots visualised with HRP chemiluminescent substrate.

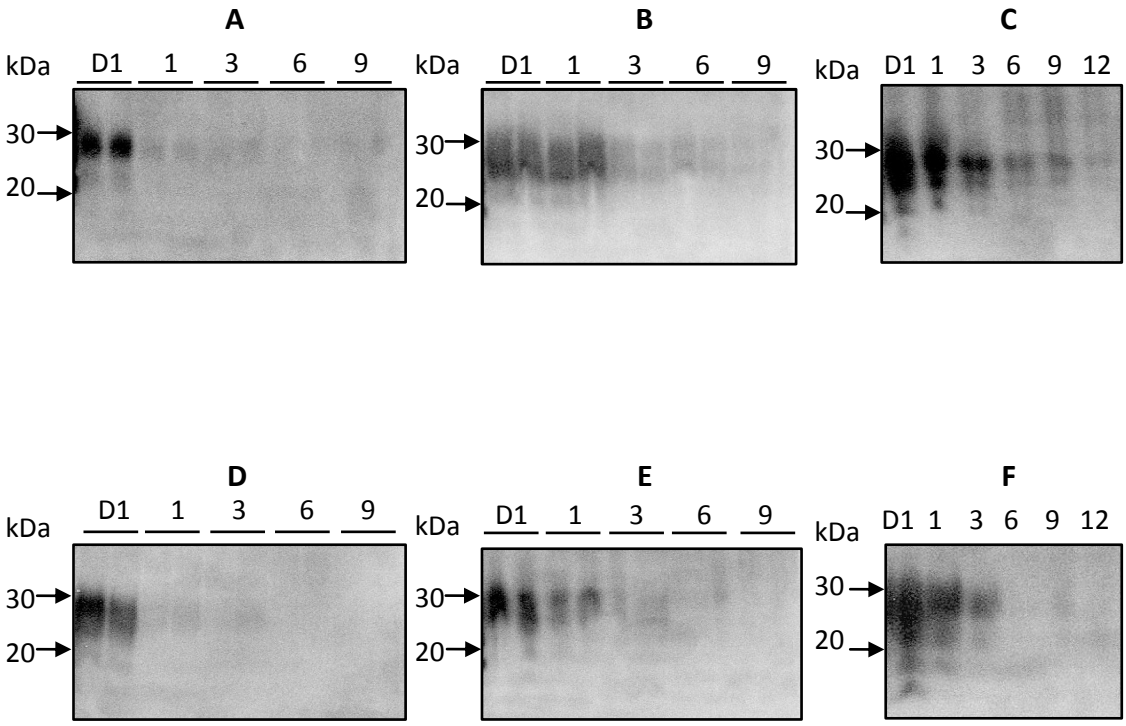


Figure 5.7. Persistence of ovine scrapie and bovine BSE PrP^{Sc} in Cambs1, Cambs2, and Hereford soils over an 18 month period. Soil samples (0.5g) of Cambs1 (panels A and D), Cambs2 (panels B and E), and Hereford (panels C and F) were extracted after 24 hours (D1), 1 month, 3 months, 6 months, 9 months, and 12 months (as indicated) co-incubation with 10µl 20% (w/v) ovine scrapie (panels A-C) or bovine BSE (panels D-F) brain homogenate per 100mg soil. Following SDS PAGE of 200mg soil equivalent samples, Western blots were probed with anti-PrP antibody SHA31 diluted 1:20,000 and anti-mouse HRP conjugated antibody, and blots visualised with HRP chemiluminescent substrate.

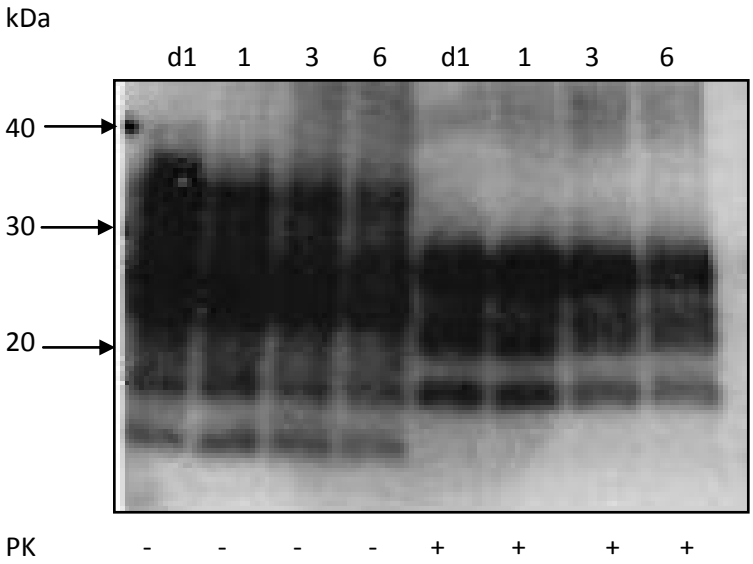


Figure 5.8. The persistence of total PrP and PrP^{Sc} over 6 months in the absence of soil. Brain homogenate (2% (w/v)) was incubated for 1 day (d1), 1 month (1), 3 months (3), and 6 months (6) as indicated at 16-20°C and either analysed directly (-) or after digestion with 10µg/ml PK for 1 hour at 37°C (+). Samples were separated on a 12% (w/v) Invitrogen NuPAGE gel and PrP detected with anti-PrP antibody SHA31 diluted 1:20,000 and anti mouse HRP conjugated antibody. Signal was visualised using HRP chemiluminescent substrate.

5.2.7. The distribution and persistence of PrP^{Sc} in soil fractionated into <63µm and >63µm fractions

In order to analyse the relative distribution of PrP^{Sc} between the "sand" component (represented by the >63µm fraction) and "silt" component (represented by the <63µm fraction) for each soil, the 6 UK soil spiked with ovine scrapie and bovine BSE brain homogenates were subjected to a fractionation procedure by passing through a 63µm sieve and the distribution of PrP^{Sc} in each fraction analysed.

Fractionation of soil was performed using homogenised soil from the entire column depth of a spiked column and was analysed by Western blotting following SDS extraction as described previously (section 2.2.13). Briefly, 1g of prion spiked soil from each of the 6 UK soils tested was separated into particles <63µm and >63µm and the entire sample from each fraction was subjected to the standard SDS extraction procedure and 20µl of this was analysed by Western blot.

There would have been differences in the actual mass of soil particles associated with each fraction due to the uneven distribution of soil particle size between soil types, i.e. the majority of soil particles in sandy soils would consist of particles >63µm and the majority of particles in clay soils would be <63µm. However, this method was chosen as it would provide data on whether PrP^{Sc} was preferentially distributed in one particular fraction for each soil, and whether this distribution varied over time.

Binding of ovine scrapie PrP^{Sc} was found to be generally either equally distributed between particle size fractions; for example with Notts1, Hereford, or Cambs3 soil, or preferentially bound to particles in excess of 63µm in the cases of Cambs1 and

Cambs2 soil (figure 5.9). In contrast, ovine scrapie PrP^{Sc} was found to bind preferentially to particles less than 63µm in the Notts2 soil despite this fraction representing only 14% by weight of the total (table 5.1). This would potentially indicate a constituent mineral or other component within that particular soil fraction with a high affinity for PrP^{Sc}.

A similar trend was observed with bovine BSE PrP^{Sc} with the exception of Hereford soil, where prion strain had an apparent effect on which fraction PrP^{Sc} preferentially bound; for ovine scrapie PrP^{Sc} there was no preferential binding of PrP^{Sc} between size fractions, while with bovine BSE PrP^{Sc} the distribution was weighted more towards soil particles in excess of 63µm (figure 5.9).

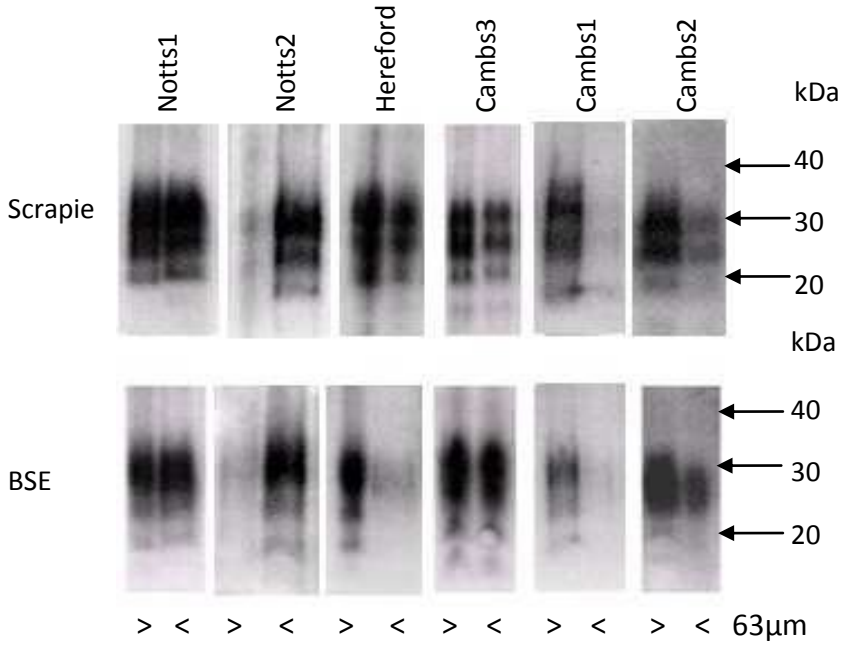


Figure 5.9. Distribution of ovine scrapie and bovine BSE PrP^{Sc} in 6 UK soils following fractionation into <63µm and >63µm fractions. Samples (1g) of Notts1, Notts2, Hereford, Cambs1, Cambs2, or Cambs3 (as indicated) spiked with 10µl 20% (w/v) ovine scrapie (upper panels) or bovine BSE (lower panels) brain homogenates for 24 hours were passed through a 63µm sieve and separated into <63µm and >63µm fractions prior to extraction with 50µg/ml PK and 10% (w/v) SDS as described previously. Extracts were methanol precipitated and, following centrifugation, resuspended in 50µl 2x Invitrogen LDS sample buffer and 20µl loaded per lane and separated by SDS PAGE. PrP^{Sc} was detected on Western blots using anti-PrP antibody SHA31 and AP conjugated anti-mouse antibody and visualised using AP chemiluminescent substrate.

5.2.8. Migration of PrP^{Sc} through soil columns over 6 month and 18 month periods

Soils spiked with ovine scrapie and bovine CNS homogenates were analysed for evidence of migration of PrP^{Sc} through the soil column over an 18 month period to determine whether PrP^{Sc} bound to soil at the top of the column was transported down the column by deionised water introduced to the top of the column every 7-14 days in order to main the soil within the columns at field moisture content. The addition of water in this manner would also mimic the vertical flow of water through soil as a result of natural precipitation within the environment. Although the addition of water in this manner to maintain field moisture content was insufficient to saturate the entire column, typically the top two thirds of the column was wetted by this procedure.

Western blot analysis was performed of PrP^{Sc} extracted from depth fractions consisting of soil collected from the top third, middle third, or bottom third of a column 24 hours and 6 months post inoculation from all six UK soils. Samples were extracted using the modified Johnson soil extraction protocol and 200mg of soil extract detected with anti-PrP antibody SHA31 (figures 5.10 and 5.11). Although samples extracted from different soils indicated differences in the initial deposition of PrP^{Sc} within the column (possibly due to different affinity interactions of prions with distinct soils and differences in soil porosity) no further migration of PrP^{Sc} was detected within the column after 6 months for the 6 soils, or for 4 of the soils which were analysed at 18 months where persistence in whole soil had been demonstrated to exceed 6 months (figures 5.12 for ovine scrapie PrP^{Sc} and figure 5.13 for bovine BSE

PrP^{Sc}). There appeared to be no correlation between prion strain and initial deposition of PrP^{Sc} with soil columns.

It should be noted that no brain homogenate was lost from the bottom of the soil columns when initial spiking was carried out, thus excluding the possibility that subsequent differences in PrP^{Sc} recovery were due to sample losses upon application of brain homogenate to the column.

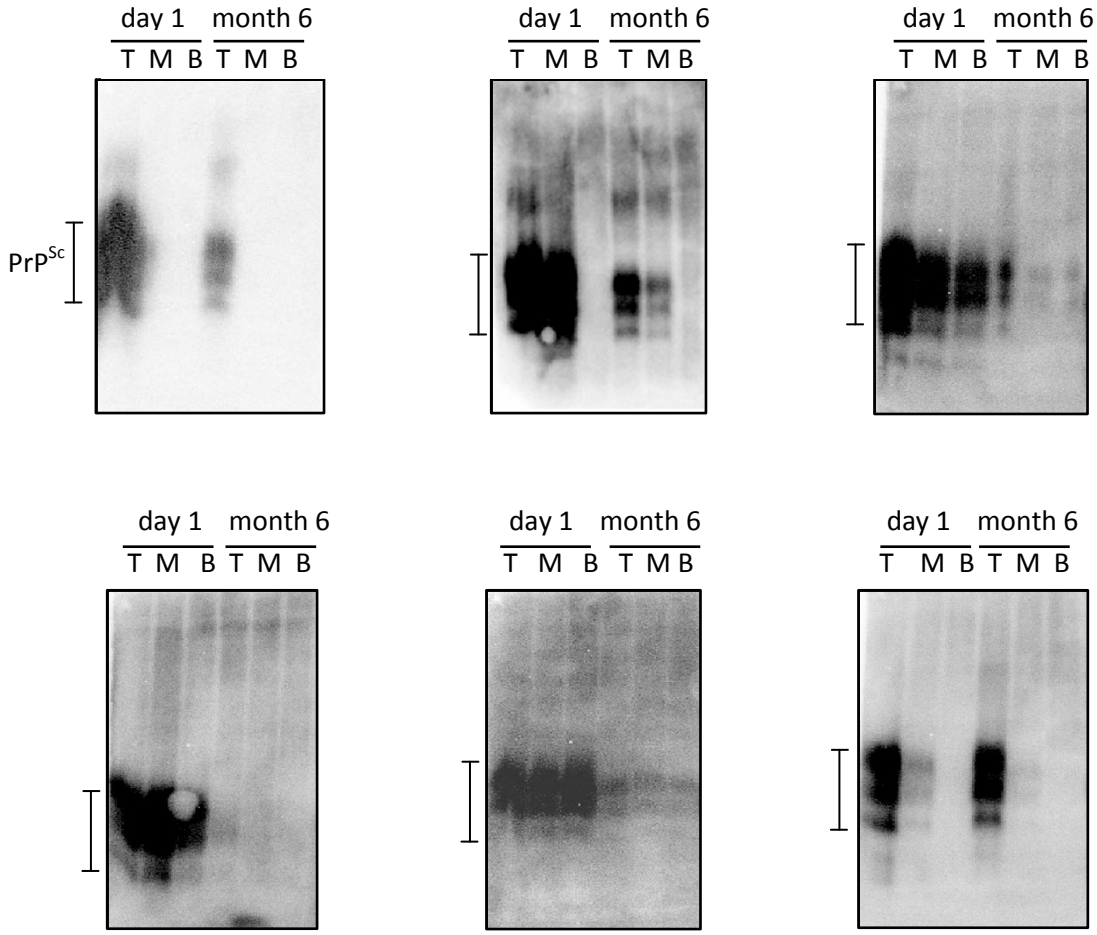


Figure 5.10. Migration of ovine PrP^{Sc} through soil columns over a 6 month period. Soil columns containing Notts1 soil (upper left panel), notts2 soil (upper centre panel), Hereford soil (upper right panel), cambs1 soil (lower left panel), cambs2 soil (lower centre panel), and cambs3 soil (lower right panel) were extracted using PK and SDS as described previously. 200mg equivalent soil extract from columns sectioned into thirds representing the top (T), middle (M), and bottom (B) fractions were analysed after 1 day and 6 months post spiking with 10µl 20% (w/v) ovine brain homogenate per 100mg soil. PrP^{Sc} (indicated by bar) was detected on Western blots using anti-PrP specific antibody SHA31 and AP conjugated anti-mouse antibody, and visualised using AP chemiluminescent substrate.

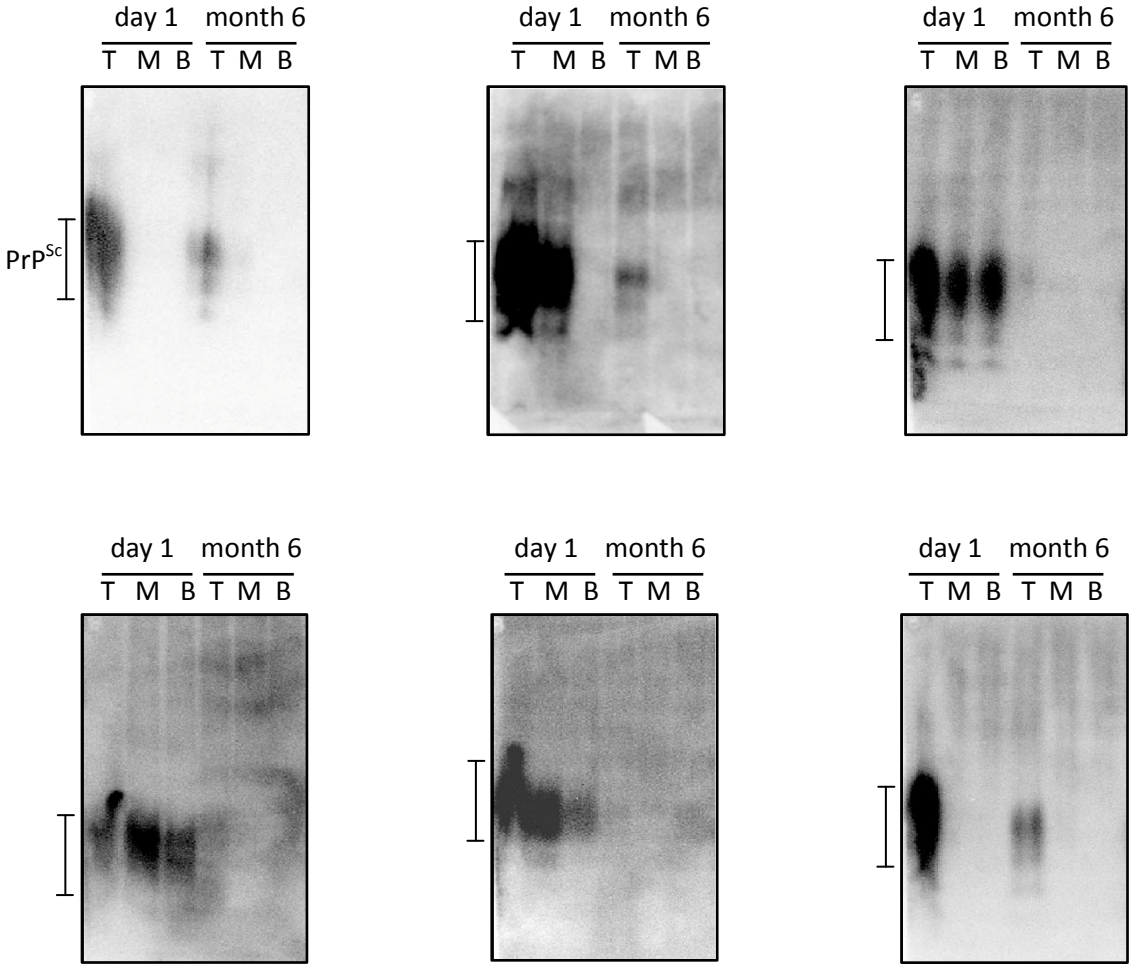


Figure 5.11. Migration of bovine PrP^{Sc} through soil columns over a 6 month period. Soil columns containing Notts1 soil (upper left panel), notts2 soil (upper centre panel), Hereford soil (upper right panel), cambs1 soil (lower left panel), cambs2 soil (lower centre panel), and cambs3 soil (lower right panel) were extracted using PK and SDS as described previously. 200mg equivalent soil extract from columns sectioned into thirds representing the top (T), middle (M), and bottom (B) fractions were analysed after 1 day and 6 months post spiking with 10µl 20% (w/v) bovine brain homogenate per 100mg soil. PrP^{Sc} (indicated by bar) was detected on Western blots using anti-PrP specific antibody SHA31 and AP conjugated anti-mouse antibody, and visualised using AP chemiluminescent substrate.

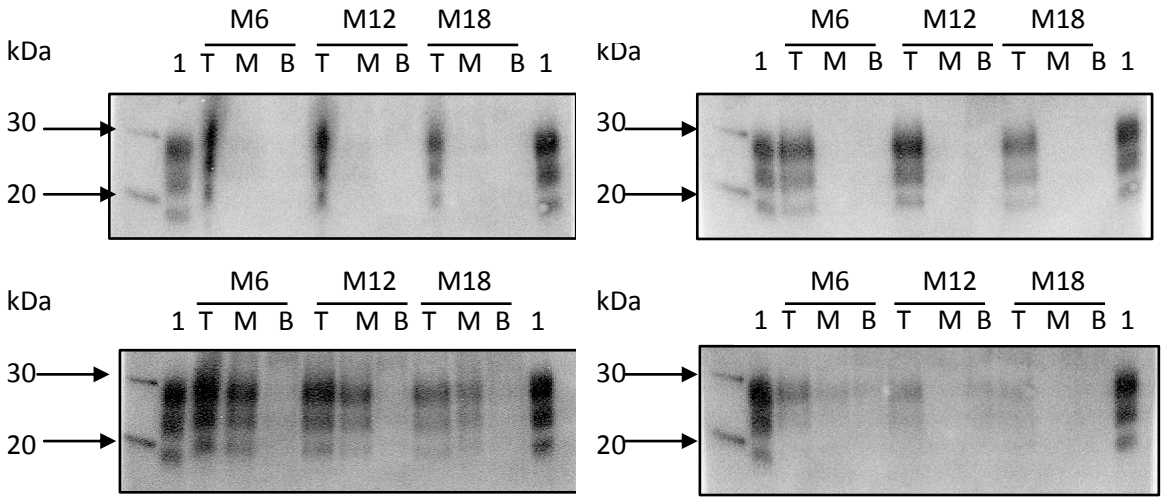


Figure 5.12. Migration of ovine scrapie PrP^{Sc} through soil columns between 6 months and 18 months post spiking as determined by Western blotting. Cambs3 soil (upper left panel), Notts1 soil (upper right panel), Notts2 soil (lower left panel) and Hereford soil (lower right panel) columns were sectioned after 6 months (M6), 12 months (M12), and 18 months (M18), and PrP extracted from the top (T), middle (M), and bottom (B) third fractions using PK and SDS as described previously. 1ul (1) of 10% (w/v) ovine brain homogenate digested with 50ug/ml PK was also loaded as indicated, and Western blots probed with SHA31 antibody and anti-mouse HRP conjugated antibody. Blots were visualised with HRP chemiluminescent substrate.

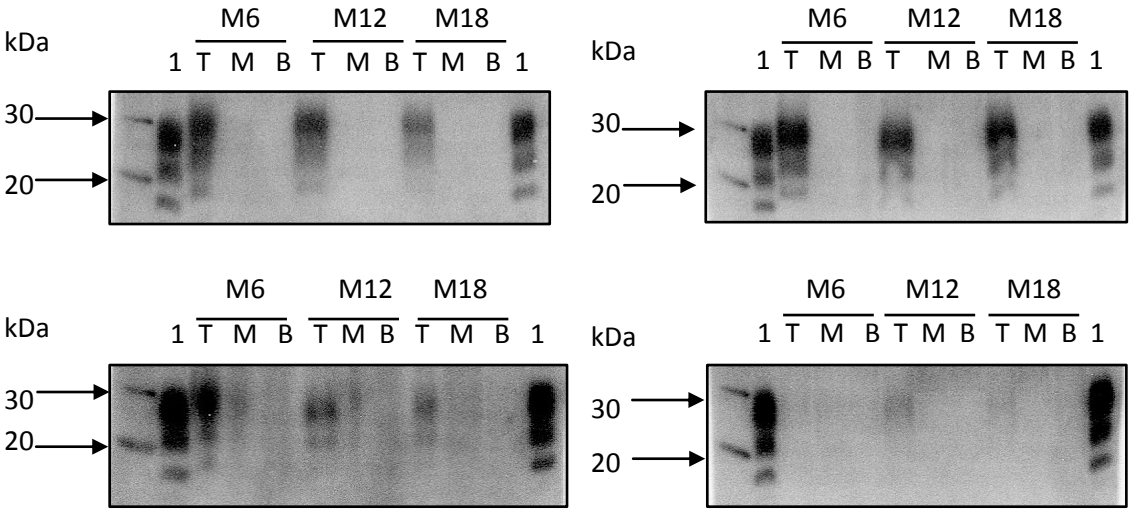


Figure 5.13. Migration of bovine BSE PrP^{Sc} through soil columns between 6 months and 18 months post spiking as determined by Western blotting. Cambs3 soil (upper left panel), Notts1 soil (upper right panel), Notts2 soil (lower left panel) and Hereford soil (lower right panel) columns were sectioned after 6 months (M6), 12 months (M12), and 18 months (M18), and PrP extracted from the top (T), middle (M), and bottom (B) third fractions using PK and SDS as described previously. 1ul (1) of 10% (w/v) bovine brain homogenate digested with 50ug/ml PK was also loaded as indicated, and Western blots probed with SHA31 antibody and anti-mouse HRP conjugated antibody. Blots were visualised with HRP chemiluminescent substrate.

5.2.9 PMCA of soil eluted prions

Protein misfolding cyclic amplification (PMCA) of PrP^{Sc} is a method that permits the rapid conversion of PrP^{C} into PrP^{Sc} using alternating cycles of incubation and sonication in a seeded reaction developed by Saborio and co-workers, and which has previously been demonstrated to be capable of the amplification of PrP^{Sc} from samples which would classically be defined as negative due to the levels of recoverable PrP^{Sc} being below the limits of detection of the Western blot procedure (Saborio *et al.*, 2001). Therefore, PMCA was performed on soil extracts following approximately 18 months co-incubation at 16-20°C with ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} in order to determine whether low levels of PrP^{Sc} were still present in those soils that were negative by Western blotting.

After 7 days incubation/amplification, samples were digested for 1 hour at 37°C with 50µg/ml PK and analysed by Western blot using monoclonal antibody SHA31. Amplification was observed using soil sample eluates from Notts1, Notts2, and Cambs2 soil (figure 5.14), while the presence of ovine scrapie PrP^{Sc} in eluates from Notts1, Notts2 and Cambs3, and bovine BSE PrP^{Sc} from Notts1 and Notts2 eluates had previously been demonstrated by Western blot at the 18 month time point (figure 5.6).

While levels of PrP^{Sc} detected by Western blot might not necessarily correlate with levels of PrP^{Sc} suitable for seeding a PMCA reaction, the absence of amplification from ovine scrapie spiked Cambs3 soil in particular may suggest that this elution method is not a universal technique for the detection of prions in the environment.

It should be noted that the presence of PK-resistant PrP^{Sc} in PMCA substrate after spiking with both ovine scrapie and bovine BSE Cambs2 soil eluates indicated that although insufficient PrP^{Sc} could be extracted using the method of Jonson and co-workers followed by Western blotting, sufficient PrP^{Sc} was eluted using the Seidel method to support PMCA amplification, and the subsequent increase in sensitivity using PMCA may permit detection of PrP^{Sc} in certain environmental soil samples over an extended time period.

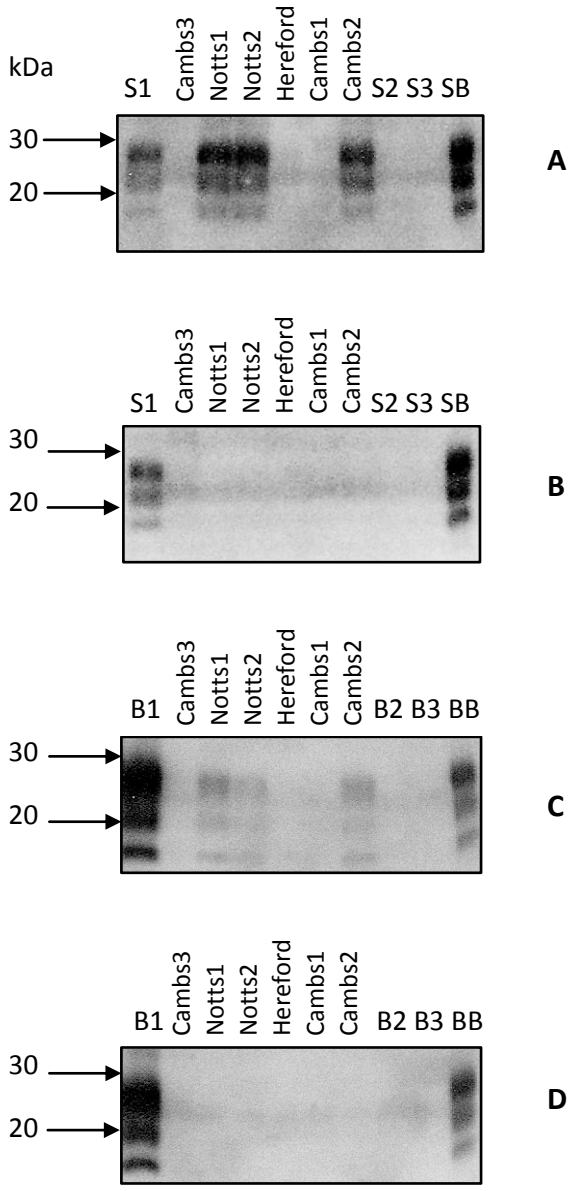


Figure 5.14. PMCA amplification of 6 UK soil extracts. PMCA was performed after incubation of soil for 18 months with 10µl 20% (w/v) scrapie ovine (panel A), healthy ovine (panel B), BSE infected bovine (panel C), or healthy bovine (panel D) brain homogenate per 100mg soil. Soil samples (100mg) were extracted from Cambs3, Notts1, Notts2, Hereford, Cambs1, and Cambs2 soil (as indicated) using 200µl of 1% (w/v) SDS and 10µl (equivalent to 5mg soil) spiked into 10% (w/v) healthy ovine (panels A and B) or bovine (panels C and D) brain homogenate and subjected to 7 days amplification as described in section 2.2.17. 6.6µl of the resulting material was digested with 50µg/ml PK for 1 hour then heated to 100°C for 10 minutes with two volumes of 2x Invitrogen LDS sample buffer and analysed by SDS PAGE along with 1µl PK digested scrapie infected ovine (SB) and BSE infected bovine (BB) brain homogenates, and scrapie PMCA (S1) and BSE (B1) positive and duplicate negative (S2, S3, and B2, B3 respectively) controls. Western blots were detected with anti-PrP antibody SHA31 and HRP conjugated anti-mouse antibody, and visualised using HRP chemiluminescent substrate.

5.3.1. The effect of environmental conditions on the retention of PrP^{Sc} on soil

Notts1 soil was selected for the analysis of environmental factors on the retention of PrP^{Sc} as data from the comparative study of UK soils had indicated that this particular soil was most capable of supporting the persistence and recovery of PrP^{Sc} when maintained at a temperature of between 16°C and 20°C over an extended time period. This factor therefore allowed the analysis of PrP^{Sc} persistence under varying environmental conditions, such as temperature, moisture content, and pH, over an extended period.

Temperatures of 25-30°C, 8-12°C, and 4°C with a monthly freeze/thaw cycle, were selected to mimic temperatures typically experienced during UK summer, spring, and winter periods respectively. Further columns were maintained at 30% below and 60% below the field moisture content at which the soil had been supplied, and columns were pH adjusted to low and high pH (pH values 4.5 and 7.6 respectively) relative to the natural pH of the soil (pH 6.2).

5.3.2. The effect of temperature on PrP^{Sc} soil retention

The elution of ovine scrapie and bovine BSE from PrP^{Sc} incubated on Notts1 soil maintained at temperature of 25°C to 30°C, 8°C to 12°C, and 4°C with a single 24 hour freezing period per month indicated that for both TSE strains PrP^{Sc} persistence was inversely correlated to temperature (figure 5.15). The highest level of persistence was observed at 4°C, with 8-12°C showing an intermediate PrP^{Sc} persistence profile, and the lowest level of persistence detected with soil columns maintained at 25-30°C. No apparent strain effect was observed with ovine scrapie PrP^{Sc} or bovine BSE PrP^{Sc} at any of the temperatures tested.

As Western blotting only provided a qualitative analysis of PrP^{Sc} persistence, PrP^{Sc} was also measured by ELISA to obtain quantitative data on the persistence of PrP^{Sc} under these temperature conditions.

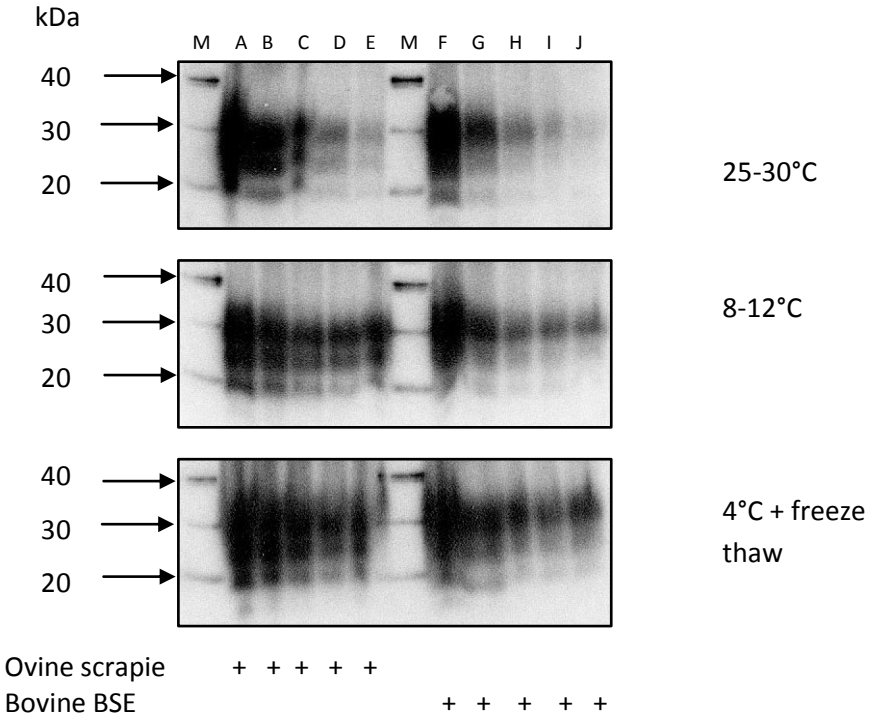


Figure 5.15. Detection of ovine scrapie and bovine BSE PrP^{Sc} after 18 months incubation on Notts1 soil at 3 temperatures. PrP^{Sc} was eluted by boiling SDS treatment following digestion on soil with 100µg/ml thermolysin for 1 hour. PrP^{Sc} from 200mg extracted soil following 1 and 4 days (lanes A and F respectively), 49 days (lanes B and G), 188 days (lanes C and H), 391 days (lanes D and I), and 566 days (lanes E and J) post inoculation were separated on 12% (w/v) Invitrogen NuPAGE gels alongside Invitrogen MagicmarkXP protein standards (M) and, following transfer to PVDF, detected using anti-PrP monoclonal antibody SHA31 and visualised using HRP chemiluminescent substrate.

PrP^{Sc} eluted from soil columns incubated at a temperature of 25-30°C was compared to PrP^{Sc} eluted from columns maintained at 8-12°C, or 4°C with monthly freeze/thaw cycles at time points of 1 day (ovine) or 4 days (bovine) , 188 days, 391 days, and 566 days post inoculation with ovine scrapie or bovine BSE CNS homogenates.

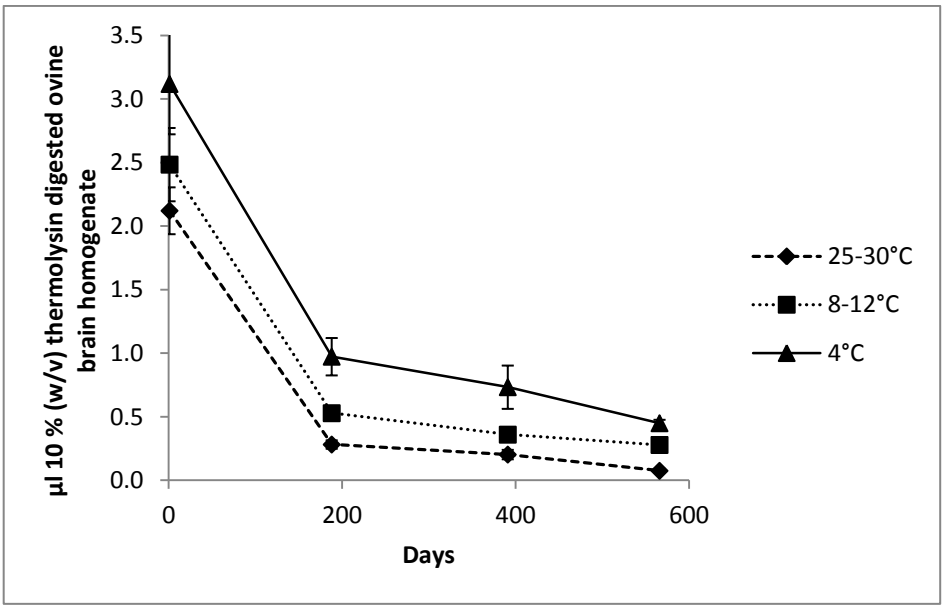
Two ELISA methods were used; a commercial TeSeE ELISA (Bio-Rad) specific for ovine scrapie PrP^{Sc} which quantified the core region of PrP^{Sc} (and therefore measured both full-length and truncated PrP^{Sc}) , and a sandwich ELISA (termed the "full-length ELISA") capable of measuring full-length PrP^{Sc} which utilised a capture antibody (SAF34) specific to the N-terminal region of PrP. Persistence was deemed to be above the limit of detection of the assay if the OD_{405nm} of the extracted sample exceeded 3X the standard deviation plus the mean OD of triplicate ELISA wells from 3 identical unspiked soil extracts.

The ELISA results confirmed Western blot data, namely that the persistence of both ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} was inversely correlated with temperature i.e. as temperature decreased PrP^{Sc} persistence increased (figure 5.16).

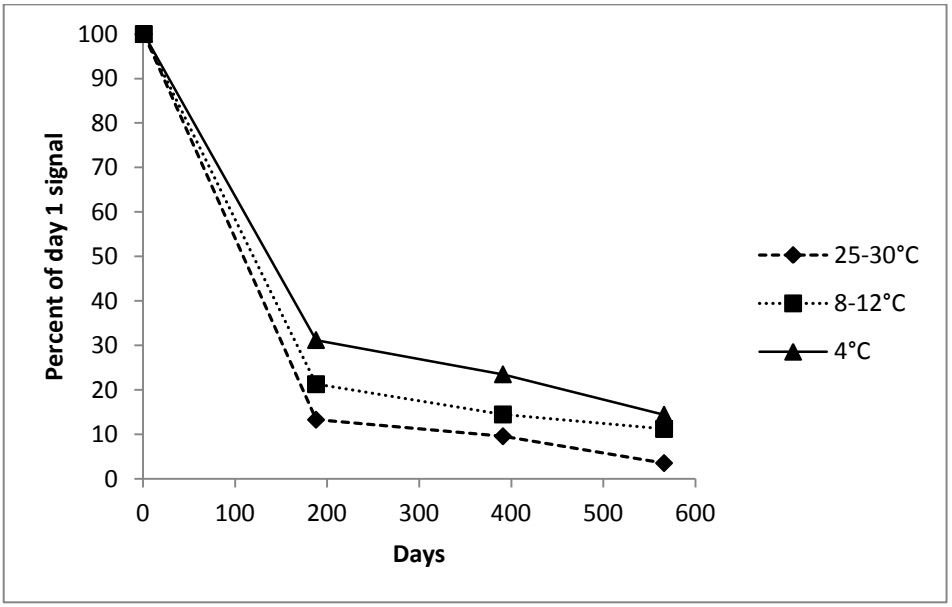
For the analysis of full-length PrP^{Sc}, samples were eluted using thermolysin and SDS. In the case of ovine scrapie PrP^{Sc}, data indicated mean recovery (all n=9) of 11.0% (SD=1.0%), 12.8% (SD=1.5%) and 16.1% (SD=2.0%) following 1 day incubation on soil at 25-30°C, 8-12°C and 4°C respectively. Over the time period of 566 days, PrP^{Sc} levels declined at all temperatures, falling below the limit of detection of the assay at 25°C at this time point, with 1.4% and 2.3% of the initial spike being eluted following incubation at 8-12°C and 4°C respectively. Full data is given in table 5.2. A two-tailed unpaired nonparametric t-test indicated a significant difference between PrP^{Sc} elution

on day 1 at 8-12°C (n=9, p=0.0216) and 4°C (n=9, p=0.0004) compared to elution at 25-30°C. Similar results were obtained after 188 days incubation at 8-12°C (n=9, p=0.0003) and 4°C (n=9, p=0.0004), and 391 days at 8-12°C (n=9, p=0.0004) and 4°C (n=9, p=0.0004). Statistical analysis of elution at 8-12°C compared to 4°C also revealed significant differences on day 1 (p=0.0004), day 188 (p=0.0003), day 391 (p=0.0004), and 566 days (p=0.0003).

While differences were observed in the levels of PrP^{Sc} recovered at the three temperatures, all data indicated similar trends in the decline of eluted PrP^{Sc} over the time course.



A



B

Figure 5.16. Determination of full-length ovine scrapie PrP^{Sc} eluted from Notts1 soil maintained at three temperatures over a 566 day period. Soil was spiked with 10µl 20% (w/v) ovine scrapie brain homogenate per 100mg soil and PrP^{Sc} was eluted using 100µg/ml thermolysin at 70°C for 1 hour followed by boiling in 10% (w/v) SDS. PrP^{Sc} signals obtained in the full-length sandwich ELISA after day 1, 188 days, 391 days, and 566 days on soil are displayed in both units of µl of 10% (w/v) thermolysin digested ovine brain homogenate (panel A), and percentage recovery relative to the signal obtained after 24 hours (panel B). PrP^{Sc} was captured on the ELISA plate using anti-PrP antibody SAF34 (specific to the N-terminal region of PrP) and detected using anti-PrP antibody SHA31 (specific to the C-terminal region). Signal was visualised using alkaline phosphatase conjugated IgG1 specific anti-mouse antibody and PNPP substrate.

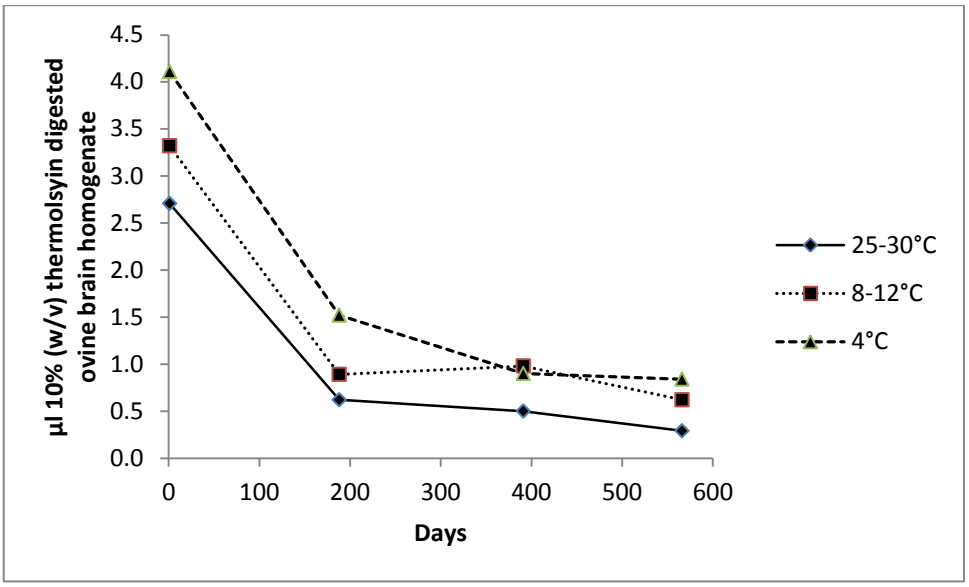
	Temperature					
	25-30°C		8-12°C		4°C	
Day	mean	SD	mean	SD	mean	SD
1	11.0%	1.0%	12.8%	1.5%	16.1%	2.0%
188	1.5%	0.2%	2.7%	0.1%	5.0%	0.8%
391	1.1%	0.2%	1.9%	0.2%	3.8%	0.9%
566	0.4%	0.1%	1.4%	0.1%	2.3%	0.1%

Table 5.2. Elution of ovine scrapie PrP^{Sc} from Notts1 soil over 566 days as determined by an ELISA which measured full-length PrP^{Sc}. Data calculated as a percentage of the initial spike.

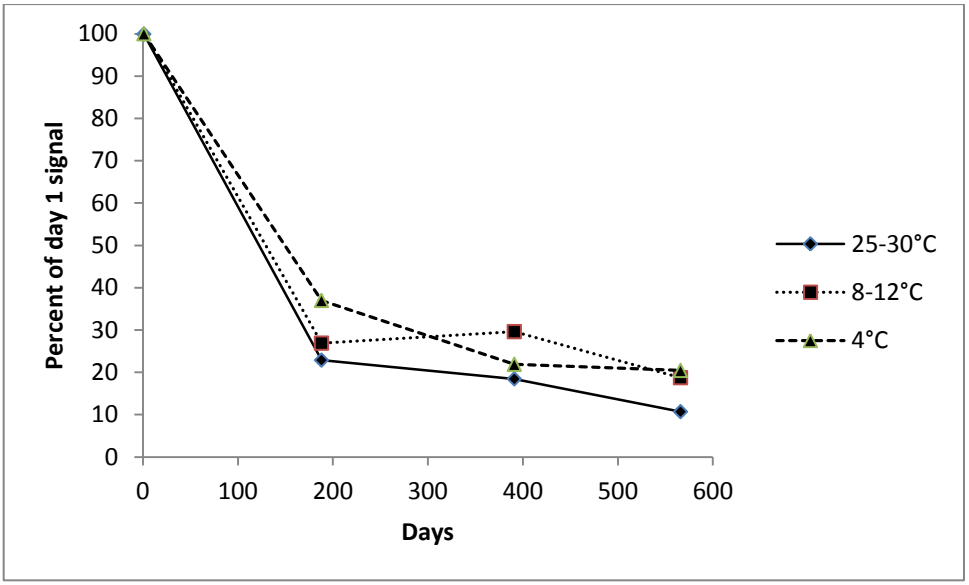
Analysis of the equivalent samples eluted using thermolysin and SDS then analysed using the Bio-Rad TeSeE ELISA kit indicated a similar profile of PrP^{Sc} persistence to the full-length-specific ELISA (figure 5.17).

The recovery of ovine scrapie PrP^{Sc} after 24 hours incubation on soil when determined using the TeSeE ELISA showed some variation compared to the figures obtained from full-length ELISA, namely recovery in the order of 2.7 to 4.1 µg/ml thermolysin digested 10% (w/v) CNS homogenate for the TeSeE ELISA and approximately 2.1 to 3.1 µg/ml thermolysin digested 10% (w/v) CNS homogenate for the full-length ELISA following 24 hours incubation on soil. This may have been representative of the desorption and subsequent detection of both full-length and truncated PrP species from the soil, as truncated PrP^{Sc} species (in addition to full-length) were measured by the TeSeE ELISA while the full-length ELISA measured only untruncated PrP^{Sc}.

Recovery of ovine scrapie PrP^{Sc} from soil maintained at 4°C for 24 hours after the initial application of brain homogenate was 21.2%, falling to 17.2% for soil maintained at 8-12°C, and 14.0% for soil maintained at 25-30°C, when analysed using the Bio-Rad TeSeE method. After 566 days incubation, recoveries were 4.3%, 3.2% and 1.5% at 4°C, 8-12°C, and 25-30°C respectively (table 5.3).



A



B

Figure 5.17. Determination of total ovine scrapie PrP^{Sc} eluted from Notts1 soil maintained at three temperatures over a 566 day period. Soil was spiked with 10µl 20% (w/v) ovine scrapie brain homogenate per 100mg soil and PrP^{Sc} was eluted using 100µg/ml thermolysin at 70°C for 1 hour followed by boiling in 10% (w/v) SDS. PrP signals obtained from single extracts were analysed using the Bio-Rad TeSeE ELISA kit after 24 hours, 188 days, 391 days, and 566 days on soil, and are displayed in both units of µl 10% thermolysin digested ovine brain homogenate (panel A), and relative to the signal obtained after 24 hours (panel B).

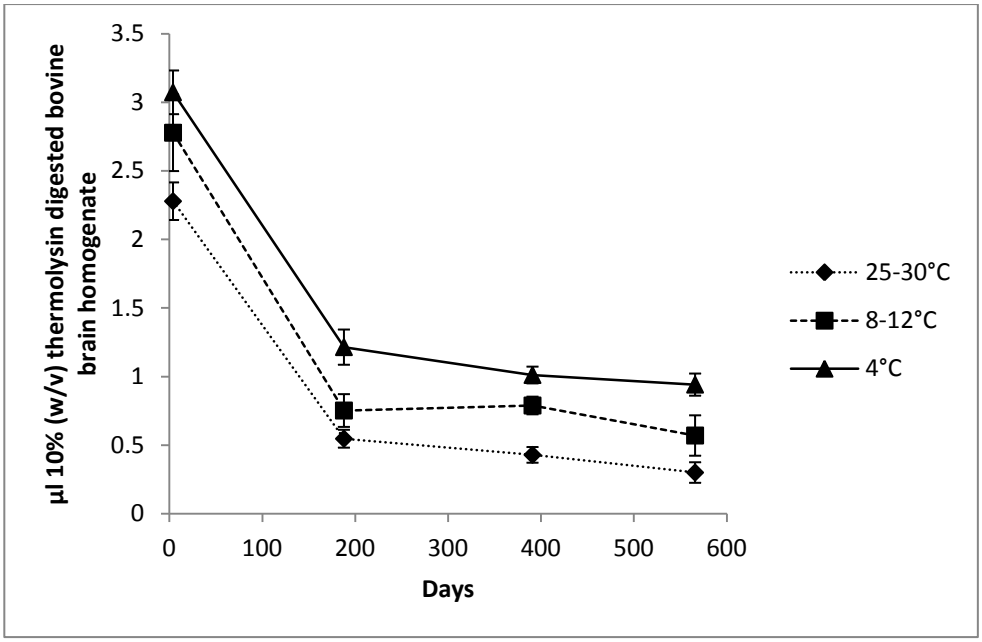
	Temperature		
Days	25-30°C	8-12°C	4°C
1	14.0%	17.2%	21.2%
188	7.1%	7.3%	9.5%
391	2.6%	5.1%	4.6%
566	1.5%	3.2%	4.3%

Table 5.3. Elution of ovine scrapie PrP^{Sc} from Notts1 soil over 566 days as determined by the Bio-Rad TeSeE ELISA. Data calculated as a percentage of the initial spike using an ELISA which measured both full-length and amino terminally truncated PrP^{Sc}. Single samples (100mg soil equivalent) were analysed.

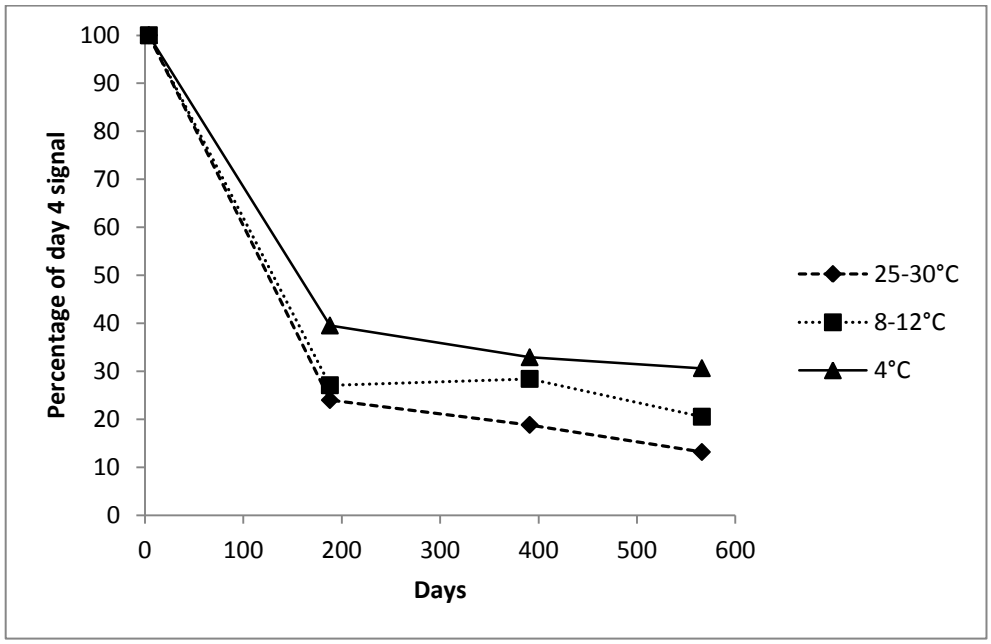
Persistence of bovine BSE PrP^{Sc} on Notts1 soil using the full-length ELISA assay indicated similar elution profiles to ovine scrapie PrP^{Sc} (figure 5.18), in that elevated temperature resulted in a reduction in the persistence of PrP^{Sc}. Triplicate analysis of triplicate soil extractions indicated recovery of bovine BSE PrP^{Sc} was 15.5% (SD 0.8%), 14.0% (SD 1.4%), and 11.5% (SD 0.7%) at 4°C, 8-12°C, and 25-30°C respectively following 4 days incubation at the indicated temperature. After 566 days incubation recoveries dropped to 4.8% (SD 0.4%), 2.9% (SD 0.7%), and 1.5% (SD 0.4%) of the initial spikes at 4°C, 8-12°C, and 25-30°C respectively. Data obtained at 566 days incubation at 25-30°C was at the limit of detection of the assay, and all data is presented in table 5.4.

Statistical analysis (two-tailed unpaired nonparametric t-test) of the elution of bovine BSE PrP^{Sc} from Notts1 soil over 566 days indicated a significant difference between elution after 4 days from soil at 8-12°C (n=9, p=0.0015) and 4°C (n=9, p=0.0004) compared to 25-30°C. Similar data was obtained following 188 days incubation at 8-12°C (p=0.0042) and 4°C (0.0004), 391 days incubation at 8-12°C (p=0.0002) and 4°C (p=0.0003), and 566 days at 8-12°C (p=0.0021) and 4°C (p=0.0003, n=9 for all data).

TeSeE ELISA analysis of thermolsyin eluted bovine BSE PrP^{Sc} was not performed due to the specificity of the ELISA kit to ovine PrP^{Sc}, however recoveries of both ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} were of a similar order and trend when analysed by full-length ELISA, confirming the absence of strain and/or host effect noted by qualitative Western blot.



A



B

Figure 5.18. Determination of full-length bovine BSE PrP^{Sc} eluted from Notts1 soil maintained at three temperatures over a 566 day period. Soil was spiked with 10µl 20% (w/v) bovine BSE brain homogenate per 100mg soil and PrP^{Sc} was eluted using 100µg/ml thermolysin at 70°C for 1 hour followed by boiling in 10% (w/v) SDS. PrP^{Sc} signals obtained in the full-length sandwich ELISA after 4 days, 188 days, 391 days, and 566 days on soil displayed are display in both unit of µl 10% (w/v) ovine brain homogenate (panel A), and percentage recovery relative to the signal obtained after 4 days (panel B). PrP^{Sc} was captured using anti-PrP antibody SAF34 (specific to the N-terminal region of PrP) and detected using anti-PrP antibody SHA31 (specific to the C-terminal region). Signal was visualised using alkaline phosphatase conjugated IgG1 specific anti-mouse antibody and PNPP substrate.

	Temperature					
	25-30°C		8-12°C		4°C	
Day	mean	SD	mean	SD	mean	SD
4	11.5%	0.7%	14.0%	1.4%	15.5%	0.8%
188	2.8%	0.3%	3.8%	0.6%	6.1%	0.6%
391	2.2%	0.3%	4.0%	0.3%	5.1%	0.3%
566	1.5%	0.4%	2.9%	0.7%	4.8%	0.4%

Table 5.4. Elution of bovine BSE PrP^{Sc} from Notts1 soil over 566 days as determined by an ELISA which measured full-length PrP^{Sc}. Data calculated as a percentage of the initial spike.

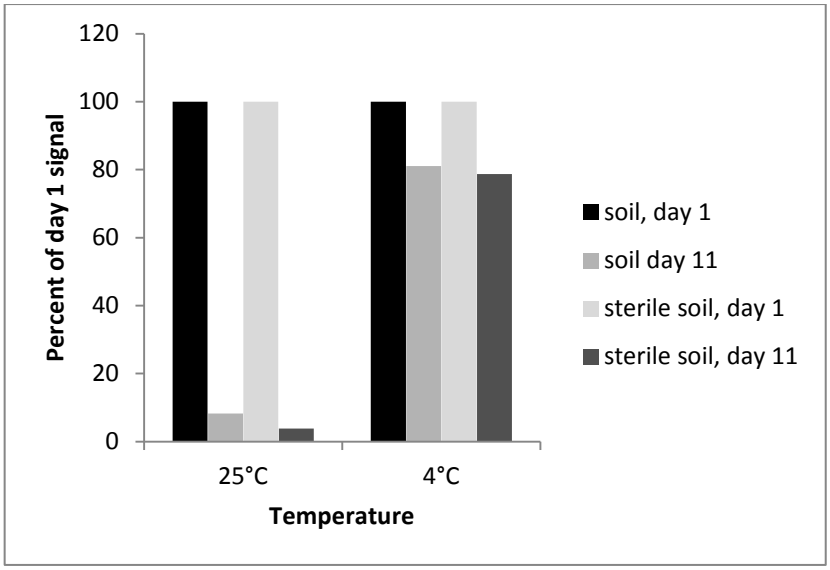
5.3.3. The effect of temperature on ovine PrP^{Sc} elution over an 11 day period

In view of the apparent rapid decline in both ovine and bovine PrP levels in soil columns during the first 49 days incubation in the presence of soil (typically a 50% reduction of the day 1 signal following 49 days incubation at 25-30°C), an analysis of ovine PrP^{Sc} recovery was performed following 1 day, and 11 days incubation at either 25°C or 4°C with 10µl 20% (w/v) ovine brain homogenate per 100mg soil. Triplicate 500mg aliquots of Notts1 soil for each condition were inoculated with 10µl 20% (w/v) CNS homogenate per 100mg and, following incubation, both soil eluate and supernatant were analysed by full-length ELISA. Both untreated and soil sterilised with a 121°C autoclave cycle for 15 minutes were analysed, to investigate whether microbial activity was influencing PrP^{Sc} recovery. Following autoclaving, soil was returned to field moisture content using sterile distilled water.

Analysis of PrP^{Sc} eluted from soil as a percentage of the day 1 signal indicated that at 25°C there was a considerable reduction in full length PrP^{Sc} compared to 4°C over the 10 day intervening period as determined by ELISA specific for the detection of full length PrP (figure 5.19). Following 11 days incubation on soil approximately 80% of the day 1 signal remained after incubation at 4°C, compared to approximately 8% of the day 1 signal at 25°C. Similar results were obtained using sterile soil, indicating that loss of the amino terminus at elevated temperatures was unlikely to be due to increased proteolytic or bacterial activity. Analysis of soil extracts from the non-sterile soils by Western blot using antibodies specific to both the N-terminal region of PrP and the protease resistant core indicated that the core region of PrP^{Sc} was still detected at relatively high levels at 25°C, and that the loss of N-terminus signal of soil

bound prion as determined by the full-length ELISA was therefore due to the elevated temperature and not due to a reduction in overall PrP^{Sc} levels (figure 5.19).

As had been observed previously, the majority of PrP^{Sc} could not be recovered despite the relatively harsh elution conditions used; recovery of full length PrP following 1 day incubation on non-sterile soil was in the order of 34% of the initial spike for soil maintained at 4°C and 18% for soil maintained at 25°C. Following 11 days incubation recoveries were 22% and 2% respectively.



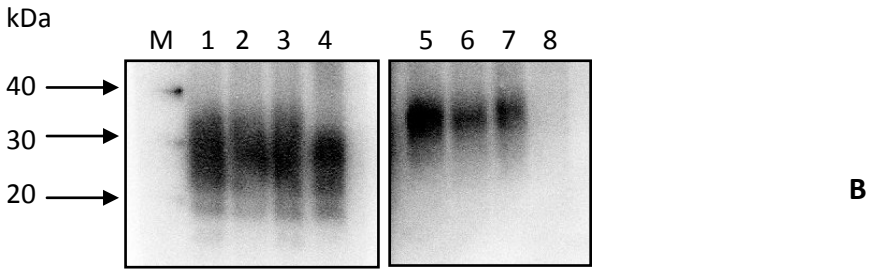


Figure 5.19. Elution of soil-bound ovine scrapie prions following 1 day and 11 days incubation at 25°C and 4°C as determined by ELISA and Western blot. 500mg aliquots of Notts1 soil were incubated with 10µl 20% (w/v) ovine scrapie brain homogenate per 100mg soil and bound PrP eluted using 100µg/ml thermolysin and 10% (w/v) SDS. Full length PrP^{Sc} (100mg extracted soil per well) was detected using a sandwich ELISA, with PrP being captured using anti-PrP antibody SAF34 and detected with anti-PrP antibody SHA31 (panel A). Signals were developed using PNPP substrate. Recovered PrP is shown as a percentage of the signal relative to that obtained following 1 day incubation on soil. Eluted PrP was further analysed by Western blot (panel B); 100mg eluted PrP was loaded per well following incubation for 1 day at 4°C (lanes 1 and 5) or 25°C (lanes 2 and 6) and 11 days at 4°C (lanes 3 and 7) or 25°C (lanes 4 and 8). PrP was detected using anti-PrP antibodies SHA31 specific to the PrP core (diluted 1:40,000) or SAF-32 specific to the N-terminal region of PrP (diluted 1:80,000). Signal was developed using HRP conjugated anti-mouse antibody diluted 1:2,000 and HRP chemiluminescent substrate.

5.3.4. The effect of pH on PrP^{Sc} soil retention

Notts1 soil was adjusted with regards to pH as described in section 2.2.9 prior to packing into columns and inoculation with CNS homogenate. The pH of Notts1 soil prior to addition of acidic or basic compounds to adjust pH was determined to be 6.2, in close agreement to the pH value of 5.9 determined by Eurofins Ltd. After the addition of ammonium sulphate or calcium carbonate pH values of aliquots of treated Notts1 soil were determined to be 4.5 and 7.6 respectively, giving a range of 3 pH units over which PrP^{Sc} persistence could be monitored. Columns were maintained at 25-30°C for the duration of the experiment.

Western blotting of soil-eluted PrP^{Sc} following a 566 day co-incubation period with ovine scrapie brain homogenate indicated no major differences in the persistence of PrP^{Sc} at the three pH values, while for bovine BSE there were only very subtle differences in the persistence of PrP^{Sc} at later time points (figure 5.20). Therefore ELISA analysis of these samples was not performed.

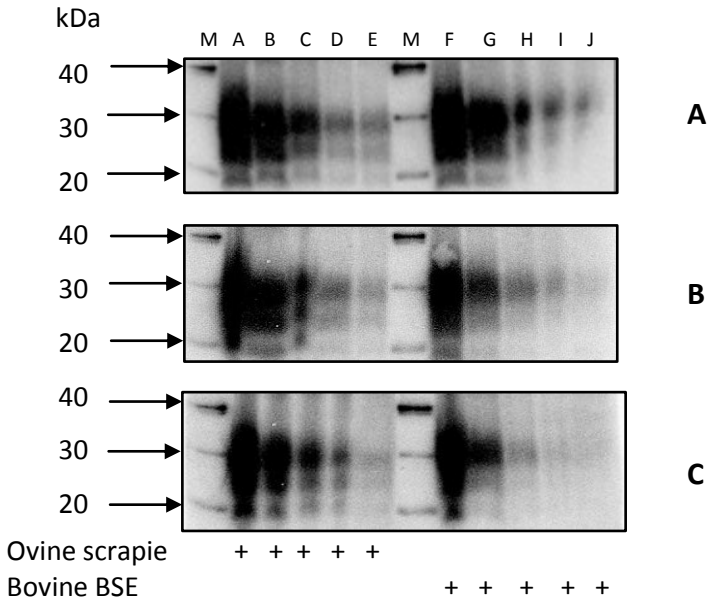


Figure 5.20. Elution of ovine scrapie and bovine BSE PrP^{Sc} over 566 days in soil adjusted to 3 pH values. PrP^{Sc} was eluted from soil adjusted to pH 4.5 (panel A), pH 6.2 (panel B), and pH 7.6 (panel C) by boiling SDS treatment following digestion on soil with 100µg/ml thermolysin for 1 hour. PrP^{Sc} from 200mg extracted soil 1 and 4 days (lanes A and F respectively), 49 days (lanes B and G), 188 days (lanes C and H), 391 days (lanes D and I), and 566 days (lanes E and J) post inoculation were separated on 12% (w/v) Invitrogen NuPAGE gels alongside Invitrogen MagicmarkXP protein standards (M) and, following transfer to PVDF, detected using anti-PrP monoclonal antibody SHA31 and visualised using HRP chemiluminescent substrate.

5.3.5. The effect of pH on prion migration

Western blot analysis of soil extracted from either the top third, middle third, or bottom third depth fractions from soil columns adjusted to pH 4.5, pH 6.2, and pH 7.6 was performed to determine whether PrP^{Sc} from ovine scrapie and bovine BSE infected brain homogenate migrated down through the soil column over the course of the analysis. As has been described previously, the moisture content of the soil columns was maintained every 7 to 14 days by the addition of deionised water at the top of the soil column to replace evaporative losses. The analysis of PrP^{Sc} migration from the soil surface down through the columns was therefore analysed as this may have implications in the field environment for the bioavailability of infectious prions deposited on the soil surface and may give an indication of the rate at which infectivity would be cleared from the soil surface to the sub soil through migration.

Analysis of soil extracts from one third fractions from soil columns by Western blotting 24 hours, 6 months, and 12 months post inoculation indicated no migration was detected during the time course (figure 5.21). Although overall PrP^{Sc} signal levels declined, there was no change in relative distribution between the fractions for either ovine scrapie or bovine BSE PrP^{Sc}.

While no change in the persistence or migration of ovine scrapie or bovine BSE PrP^{Sc} was observed at the 3 defined pH values used, distribution of PrP^{Sc} within the soil columns upon inoculation with TSE infected CNS homogenate did appear to be dependent on pH (figure 5.21). For ovine scrapie, PrP^{Sc} was found mostly in the upper third fraction at pH 4.5 with only very low levels of PrP^{Sc} in the middle fraction at this pH. At higher pH values PrP^{Sc} appeared to be evenly distributed between the upper

third and middle third fractions. This difference was more pronounced with bovine BSE PrP^{Sc} (figure 5.22), with no detectable PrP^{Sc} in the middle third fraction at pH 4.5, intermediate levels of PrP^{Sc} in the middle fraction at pH 6.2, and high levels of PrP^{Sc} in the middle fraction at pH 7.6. These results were representative of multiple column analyses, however it should be noted that on occasion the distribution of PrP^{Sc} at pH 4.5 and pH 6.2 demonstrated similar patterns. However, data was consistent that at above neutral pH penetration of PrP^{Sc} was more pronounced than at acidic pH values.

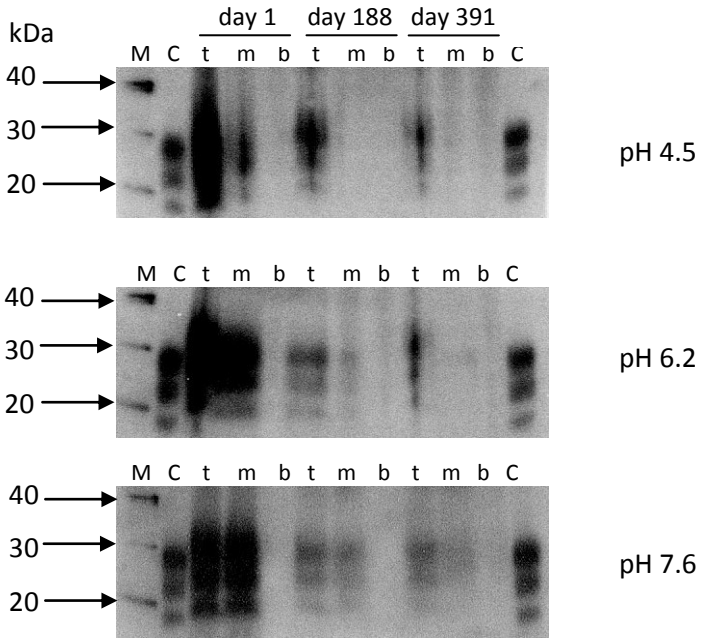


Figure 5.21. Migration of ovine scrapie PrP^{Sc} over a 391 day period through soil columns at three pH values. Soil extracts corresponding to 200mg soil from top third (t), middle third (m), and bottom third (b) depth fractions prepared after 24 hours, 188 days and 391 days co-incubation on soil were loaded on Invitrogen 12% NuPAGE protein gels. Invitrogen MagicmarkXP (M) and control loadings (C) of 1µl of 10%(w/v) ovine brain homogenate digested for 1 hour with 50µg/ml proteinase K were also loaded, and proteins transferred to PVDF membrane following electrophoresis. PrP^{Sc} was detected using anti-PrP monoclonal antibody SHA31 and anti-mouse HRP conjugated antibody and visualised using HRP chemiluminescent substrate

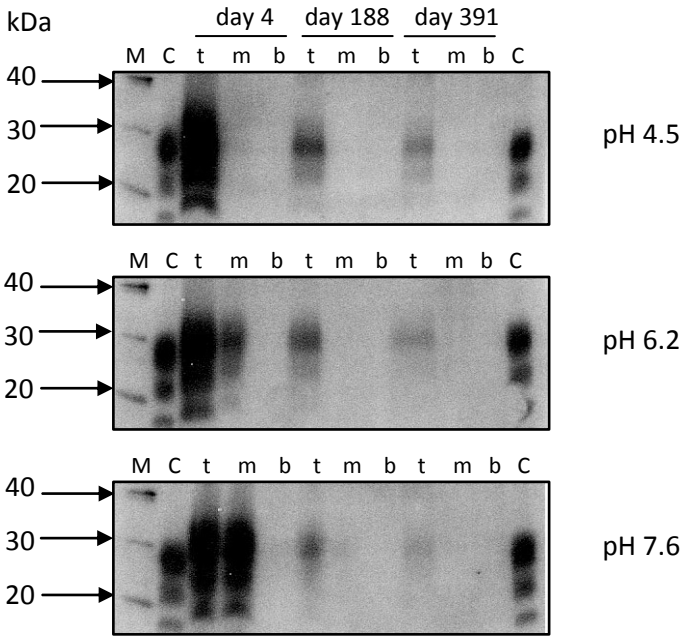


Figure 5.22. Migration of bovine BSE PrP^{Sc} over a 391 day period through soil columns at three pH values. Soil extracts corresponding to 200mg soil from top third (t), middle third (m), and bottom third (b) depth fractions prepared after 4 days, 188 days and 391 days co-incubation on soil were loaded on Invitrogen 12% NuPAGE protein gels. Invitrogen MagicmarkXP (M) and control loadings (C) of 1µl of 10%(w/v) ovine brain homogenate digested for 1 hour with 50µg/ml proteinase K were also loaded, and proteins transferred to PVDF membrane following electrophoresis. PrP^{Sc} was detected using anti-PrP monoclonal antibody SHA31 and anti-mouse HRP conjugated antibody and visualised using HRP chemiluminescent substrate.

5.3.6. Elution of prion following 1 and 4 hours incubation on Notts1 soil at pH 4.5 to pH 9.4

Given the apparent pH dependent deposition of PrP^{Sc} within the soil columns upon initial application of the brain homogenate as investigation was made into the elution of ovine scrapie PrP^{Sc} over a time period of 1 and 4 hours at the 3 soil pH values already used (pH 4.5, 6.2, and 7.6) and a higher soil pH value (pH 9.4) achieved via the addition of additional calcium carbonate to the pH 7.6 Notts1 soil.

Single extractions of 500mg Notts1 soil spiked with 10µl 20% (w/v) scrapie infected ovine brain homogenate per 100mg soil for either 1 or 4 hours co-incubation were performed. Both soil-associated PrP^{Sc} (eluted with thermolysin and SDS) and unbound PrP^{Sc} in the supernatant were analysed by ELISA measuring full-length PrP^{Sc}.

Recovery of full length ovine PrP^{Sc} as monitored by full-length ELISA ranged from approximately 14% of the spike at pH 4.5 after 1 hour binding, falling to approximately 10% of the spike at pH 9.4. Following 4 hours co-incubation of ovine brain homogenate with pH adjusted soil, recovery ranged from approximately 18% at pH 4.5 to approximately 9% at pH 9.4 (figure 5.23). PrP^{Sc} recovered by methanol precipitation of thermolysin digested supernatant was below 5% of the initial spike at all time points and pH values with the exception pH 9.4, whereby 7.5% of the initial spike was recovered after 1 hour and 10.3% after 4 hours co-incubation. Total PrP^{Sc} (the sum of recovered PrP^{Sc} from both the soil extraction procedure and the supernatant, did not exceed 20% of the initial spike under any conditions tested, suggesting the majority of PrP^{Sc} was irrecoverable (table 5.5).

At both 1 hour and 4 hours co-incubation the elution of PrP^{Sc} from the soil fraction was elevated at acidic pH and reduced at more basic pH values. In contrast, at elevated pH values recovery of PrP^{Sc} from the supernatant was higher than at low pH values i.e. the binding and elution of PrP^{Sc} from soil was inversely correlated to pH.

This data suggests that the binding of ovine scrapie PrP^{Sc} to soil occurs most rapidly at lower pH values and is slower at high pH values, and while the majority of the PrP^{Sc} was not eluted at any pH value tested, soil pH did have an effect on the binding and elution of ovine scrapie PrP^{Sc} following short term incubation on soil. This data was in agreement with the deposition profiles seen upon spiking of ovine scrapie PrP^{Sc} onto soil columns containing Notts1 soil at defined pH values.

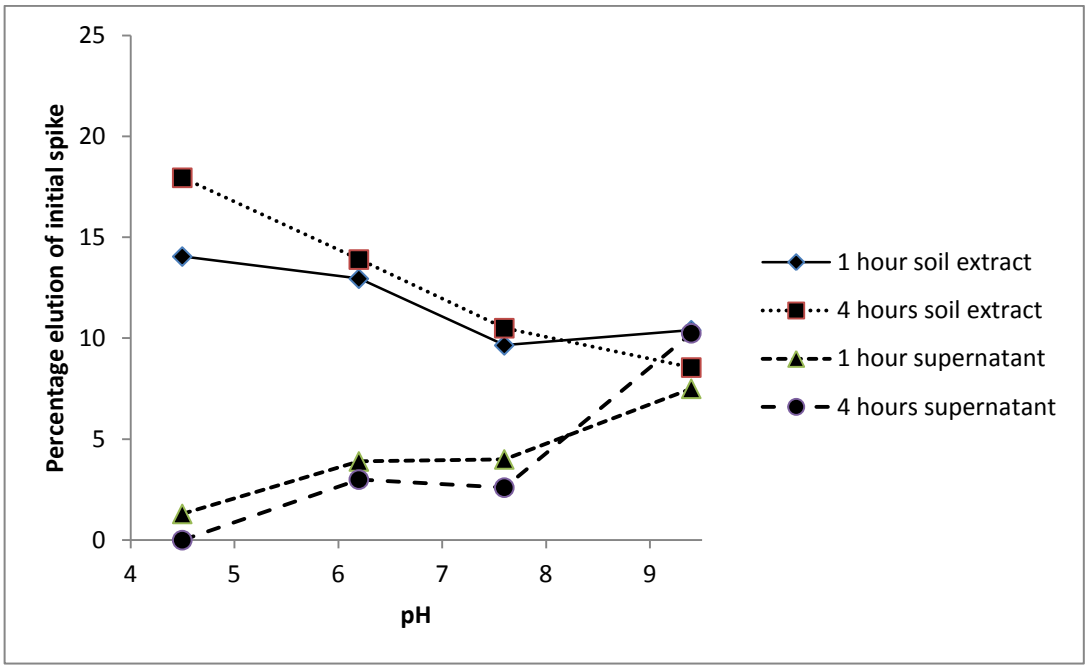


Figure 5.23. ELISA of ovine PrP^{Sc} eluted from Notts1 soil at 4 pH values following 1 hour and 4 hours co-incubation. 500mg Notts1 soil adjusted to the appropriate pH (4.5, 6.2, 7.6, 9.4) with ammonium sulphate or calcium carbonate was spiked with 10µl 20% (w/v) ovine scrapie brain homogenate per 100mg soil and incubated for 1 hour and 4 hours at room temperature (21°C). PrP^{Sc} was eluted using thermolysin and boiling SDS as described previously and 100mg soil-equivalent analysed using a sandwich ELISA capable of detecting full-length PrP^{Sc}. Soil supernatant (100mg soil equivalent) was thermolysin digested to remove PrP^{C} prior to methanol precipitation, and PrP^{Sc} recovered by centrifugation. PrP^{Sc} was visualised with alkaline phosphatase conjugated IgG1 specific anti-mouse antibody and PNPP substrate and signal intensity monitored at 405nm.

time	pH			
1 hour	**4.5**	**6.2**	**7.6**	**9.4**
soil extract	14.0%	13.0%	9.7%	10.4%
supernatant	1.3%	3.9%	4.0%	7.5%
total recovery	15.3%	16.9%	13.7%	17.9%
4 hours				
soil extract	18.0%	13.9%	10.5%	8.6%
supernatant	0%	3.0%	2.6%	10.3%
total recovery	18.0%	16.9%	13.1%	18.9%

Table 5.5. Elution of ovine scrapie PrP^{Sc} following binding to Notts1 soil for 1 and 4 hours at pH 4.5, 6.2, 7.6, and 9.4. Soil adjusted with either ammonium sulphate or calcium carbonate was spiked with 10µl 20% (w/v) ovine scrapie brain homogenate per 100mg soil for 1 and 4 hours prior to elution of PrP^{Sc} and analysis by an ELISA which measured full length PrP^{Sc}. Recoveries are shown as a percentage of the initial spike.

5.3.7. The effect of moisture on prion retention

Soil columns were prepared to standard field moisture content, 30% below field moisture content (based on dry weight of soil), and 60% below field moisture content prior to inoculation with ovine scrapie or bovine BSE brain homogenates and subsequent maintenance at 25°C to 30°C for 566 days. PrP^{Sc} eluted from the soil using the modified Johnson protocol was analysed by Western blotting and ELISA.

In contrast to results obtained for Notts1 soil maintained at 3 temperature regimes, a comparison of PrP^{Sc} levels eluted from 200mg of soil using 100µg/ml thermolysin and 10% (w/v) SDS by Western blotting indicated that the moisture content of the soil did not appear to affect relative levels of PrP^{Sc} in the eluates (figure 5.24). Furthermore, TSE strain effects were also absent in that PrP^{Sc} levels during the 566 day co-incubation on soil were comparable between ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc}.

The persistence of ovine scrapie PrP^{Sc} was, however, monitored by full-length ELISA over the 566 day co-incubation, with ELISA data adjusted to equalise soil content (based on wet weight of soil) to account for differences in the soil:water ratio in each 500mg soil aliquot of samples maintained at -30% and -60% FMC relative to FMC samples.

The recovery of ovine scrapie PrP^{Sc} from Notts1 soil after 24 hours co-incubation on soil subsequently maintained at field capacity was approximately 11.0% (SD 0.3%), increasing to approximately 13.4% (SD 0.3%) for soil maintained at 30% below field capacity, and 14.9% (SD 1.7%) for soil maintained at 60% below field capacity (figure 5.25 and table 5.6). After 391 days incubation on soil recoveries of PrP^{Sc} from all three

conditions was 1% or less of the initial spike and below the limit of detection of the assay.

Statistical analysis (unpaired non-parametric two-tailed t-test) on day 1 samples indicated a significant difference between the field moisture content elution of PrP^{Sc} and -30% FMC (n=9, p=0.0004), and -60% FMC (n=9, p=0.0004). Less significant difference was detected between -30% FMC and -60% FMC on day 1 (n=9, p=0.0168). After 188 days incubation on soil no significant difference was detected between the field moisture content elution of PrP^{Sc} and -30% FMC (n=9, p=0.2571), but was significant at -60% FMC (n=9, p=0.0004) compared to FMC. A comparison of -30% FMC and -60% FMC at 188 days also indicated a significant difference (n=9, p=0.0004). Statistical analysis was not performed for PrP^{Sc} elution after 391 or 566 days as the data was below the limit of detection of the assay, however the trends in PrP^{Sc} persistence were similar at each of the three moisture contents tested.

In view of the limited ability of the assay to monitor persistence after 391 days determination of the elution of bovine BSE PrP^{Sc} from soils maintained at different moisture contents was not performed. However, from Western blot data bovine BSE PrP^{Sc} persistence on Notts1 soil followed a similar trend to that of ovine scrapie PrP^{Sc} i.e. there were no apparent strain differences in the persistence of the two prion strains tested on Notts1 soil as a result of moisture content of the soil.

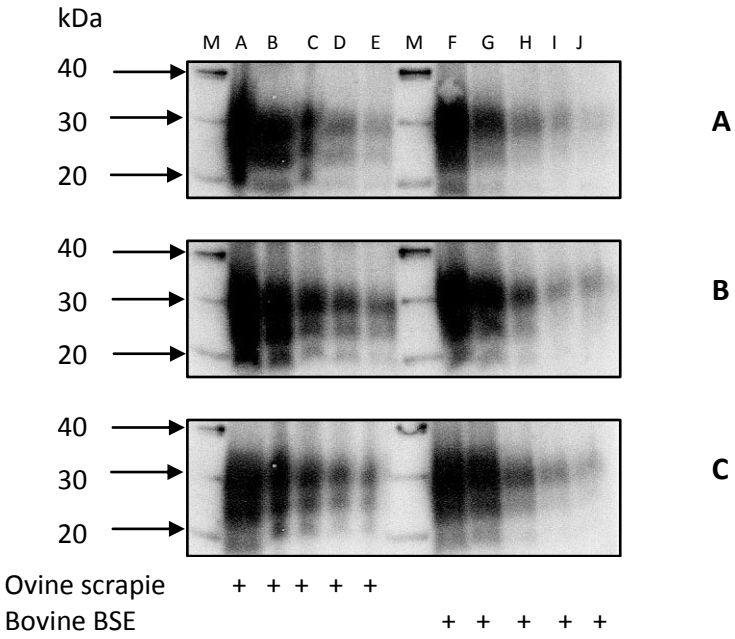
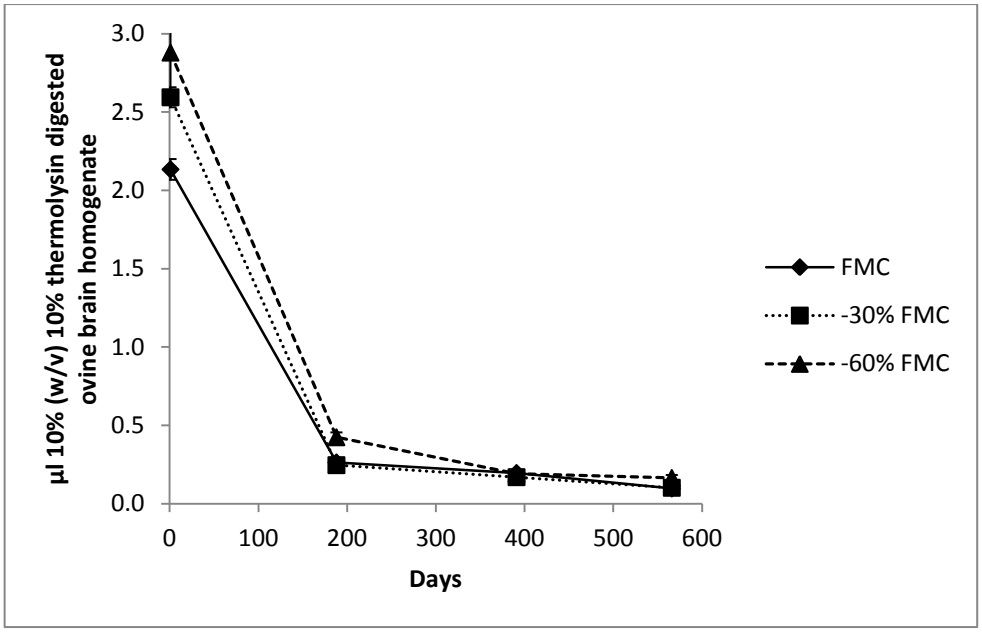
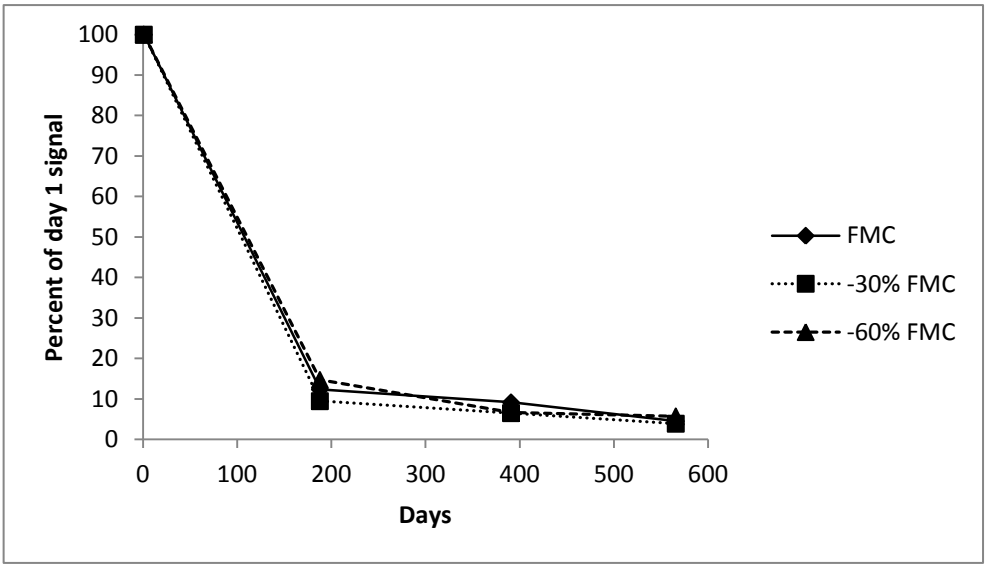


Figure 5.24. Persistence of ovine scrapie and bovine BSE PrP^{Sc} over 566 days in soil adjusted to 3 moisture contents. PrP^{Sc} was eluted from soils maintained at field moisture content (panel A), 20% below field moisture content (panel B), and 40% below field moisture content (panel C) by boiling SDS treatment following digestion on soil with 100µg/ml thermolysin for 1 hour. PrP^{Sc} eluted from 200mg extracted soil was analysed following 1 and 4 days (lanes A and F respectively), 49 days (lanes B and G), 188 days (lanes C and H), 391 days (lanes D and I), and 566 days (lanes E and J) co-incubation with ovine scrapie or bovine BSE CNS homogenates where indicated, and were separated on 12% (w/v) Invitrogen NuPAGE gels alongside Invitrogen MagicmarkXP protein standards (M) and, following transfer to PVDF, detected using anti-PrP monoclonal antibody SHA31 and visualised using HRP chemiluminescent substrate.



A



B

Figure 5.25. Determination of full-length ovine scrapie PrP^{Sc} eluted from Notts1 soil maintained at three moisture contents over a 566 day period. Soil was spiked with 10µl 20% (w/v) ovine brain homogenate per 100mg soil and PrP^{Sc} was eluted using 100µg/ml thermolysin at 70°C for 1 hour followed by boiling in 10% (w/v) SDS. PrP signals obtained in the full-length sandwich ELISA after 24 hours, 188 days, 391 days, and 566 days on soil are both in units of µl thermolysin digested brain homogenate (panel A) and displayed relative to the signal obtained after 24 hours (panel B). PrP^{Sc} was captured using anti-PrP antibody SAF34 (specific to the N-terminal region of PrP) and detected using anti-PrP antibody SHA31 specific to the C-terminal region. Signal was visualised using alkaline phosphatase conjugated IgG1 specific anti-mouse antibody and PNPP substrate.

	moisture content					
	FMC		-30% FMC		-60% FMC	
Day	mean	SD	mean	SD	mean	SD
1	11.0%	0.3%	13.4%	0.3%	14.9%	1.7%
188	1.4%	0.1%	1.3%	0.2%	2.2%	0.2%
391	1.0%	0.1%	0.9%	0.1%	1.0%	0.1%
566	0.5%	0.0%	0.5%	0.0%	0.8%	0.1%

Table 5.6. Elution of ovine scrapie PrP^{Sc} from Notts1 soil over 566 days as determined by an ELISA which measured full-length PrP^{Sc}. Data calculated as a percentage of the initial spike.

5.4. Mouse bioassay of the persistence of infectivity of Notts1 soil

In order to determine the infectivity of Notts1 soil following co-incubation at 25-30°C with ovine scrapie or bovine BSE brain homogenates, 100mg aliquots were taken from soil columns following 1 day (ovine) or 4 days (bovine) and 218 days post spiking and PrP^{Sc} extracted as described in section 2.2.16. Whilst data had been obtained on the elution efficiency of PrP^{Sc} from this soil by Western blot and ELISA, the mouse bioassays were performed in order to assess whether levels of infectivity corresponded to levels of eluted PrP^{Sc}.

All mouse bioassays were performed by R. Lockey and C. Vickery (VLA, UK), and were carried out under project and personal license issued in accordance with national guidelines.

It was originally proposed that mice would be inoculated orally by mixing 100mg of ovine scrapie or bovine BSE spiked whole soil with jam or honey to increase palatability. Initial data provided by the VLA indicated that wild-type mice could be persuaded to consume unspiked soil in this manner with no detrimental effects. It was initially planned to test oral infectivity of both ovine scrapie spiked soil extracts in Tg338 mice (transgenic for ovine VRQ PrP) and bovine BSE spiked soil in TgShpXI (transgenic for ovine ARQ PrP and susceptible to BSE infection) mice. However, tests determined that TgShpXI mice proved refractory to BSE infection at high oral dose of brain homogenate; at 10^{-1} and 10^{-2} dilutions as no animals became infected with the bovine brain homogenate pool used in this study (10 animals per dilution) by 602 days post inoculation (table 5.7). Intracerebral (IC) inoculation of five TgShpXI mice using a 10^{-1} dilution of bovine BSE homogenate resulted in 5/5 mice symptomatic at an

average of 359 days post inoculation (PI). An alternative mouse line, Tg110 (transgenic for bovine PrP), was proposed and obtained by the VLA but difficulty was encountered in establishing a breeding colony due to pups being eaten soon after birth.

Oral inoculation of female Tg338 mice with 10^{-1}, 10^{-2}, and 10^{-3} dilutions of ovine scrapie brain homogenate pool used for soil spiking experiments in this work resulted in 6/10 mice symptomatic at an average of 544 days PI at 10^{-1} dilution. Three further animals presented with disease at an average of 456 days PI following inoculation with a 10^{-2} dilution of brain homogenate.

IC inoculation of Tg338 mice resulted in 10/10 symptomatic animals at 10^{-3} dilution with a mean incubation period of 85 days, and 10/10 symptomatic at 10^{-4} at a mean of 108 days. One further mouse was positive at 10^{-5} dilution 526 days PI, and one at 10^{-6} dilution 672 days PI. These results suggest that while this transgenic line is very sensitive to IC challenge with prions, oral susceptibility is relatively low. In light of these results, Tg338 mice were inoculated by IC using PrP^{Sc} extracted from soil as described in section 2.2.16.

For the analysis of infectivity in soil extracts following IC inoculation, ten Tg338 mice were tested per condition with a 10µl (100mg soil equivalent) intracerebral injection per animal. Soil extracts prepared 24 hours post spiking on soil and incubated at 25-30°C resulted in 8 of 10 animals developing disease symptoms after an average of 130 days post inoculation. One animal were asymptomatic after 243 days post inoculation, and one further animal developed symptoms after 483 days post inoculation. No disease symptoms were detected in mice inoculated with soil extract prepared following 218 days co-incubation of soil with ovine scrapie brain homogenate at 25-

30°C at the time of writing of this work (581 days post inoculation), although it has been established that infectivity was maintained following 24 hours incubation on Notts1 soil and that the method chosen for eluting disease-associated PrP^{Sc} was successful. This work is currently ongoing by the VLA and final results are expected in spring 2012.

murine BSE titration results

TSE	inoculation	dilution	mouse strain	Histology result/days PI										Age of live mice in days PI
BSE	Oral	10^{-1}	TgShpXI	217	299	371	481	491	497	532	543	+/-	602	
BSE	Oral	10^{-2}	TgShpXI	48	307	312	391	398	398	398	411	426	437	
BSE	IC	10^{-2}	TgShpXI	243	350	384	410	410						

murine scrapie titration results

scrapie	Oral	10^{-1}	Tg338	116	324	450	463	637	664	667	693	750	778	
scrapie	Oral	10^{-2}	Tg338	119	297	305	534	689	710	717	780	839	851	
scrapie	Oral	10^{-3}	Tg338	289	664	716	723	743	779	819	832	937	937	
scrapie	IC	10^{-3}	Tg338	82	82	82	82	85	85	85	85	89	89	
scrapie	IC	10^{-4}	Tg338	90	93	94	96	100	100	108	119	134	146	
scrapie	IC	10^{-5}	Tg338	405	526	600	A	A	A	A	A	A	A	727
scrapie	IC	10^{-6}	Tg338	453	613	672	705	717	728	770	800	900	904	
scrapie	IC	10^{-9}	Tg338	127	483	523	572	701	729	853	853	894	894	

soil samples

Notts1 day 1	IC	neat	Tg338	113	118	124	124	124	129	138	167	243	483	
Notts1 day 218	IC	neat	Tg338	A	A	A	A	A	A	A	A	A	A	581

222	positive result
222	negative result
A	alive
AR	awaiting result
+/-	inconclusive result
222	positive result IHC only

Table 5.7. Current status (as of November 2011) of mouse bioassay to determine infectivity of PrP^{Sc} eluted from Notts1 soil. TgShpXI or Tg338 mice were characterised for TSE status following IC or oral inoculation with serial dilutions of ovine scrapie brain or bovine BSE 20% (w/v) CNS homogenate, or 100mg Notts1 soil extract following 1 or 218 days co-incubation at 25-30°C with 9.7µl 20% (w/v) ovine scrapie CNS homogenate.

Data produced by R. Lockey and C. Vickery with TSE diagnosis based on IHC and vacuolation scoring as described in section 2.2.16.

CHAPTER 6

Discussion

6.1 The use of thermolysin for the clearance of PrP^{C} and the production of full-length PrP^{Sc}.

This work describes the novel use of the protease thermolysin to generate PrP^{Sc} free from contaminating PrP^{C} from both scrapie and BSE infected CNS material. In contrast to proteinase K, the prototypical protease used for the clearance of PrP^{C} and generation of protease-resistant PrP^{Sc}, thermolysin digestion results not only in the rapid clearance of PrP^{C} (and the majority of other brain proteins) from both ovine and bovine CNS homogenates, but also the generation of full-length PrP^{Sc} with an intact N-terminal region. The presence of full-length PrP in thermolysin digested brain homogenate was confirmed through the use of an anti-PrP antibody specific to the N-terminal region and a Western blot migration profile identical to that obtained for total PrP. The use of thermolysin digestion as a technique for the rapid generation of full-length PrP^{Sc} free from PrP^{C} contamination compares favourably to previously published methods requiring multiple centrifugation steps in conjunction with gel filtration chromatography (Hope *et al.*, 1986; Hope *et al.*, 1988)

Thermolysin is a thermostable metalloprotease produced from the gram positive bacterium *Bacillus thermoproteolyticus* of approximately 37.5kDa (Endo, 1962). Previous uses have included the analysis of thermal unfolding of RNase A (Arnold *et al.*, 1996) and protein mass mapping (Bark et al, 2001). It has been predicted to cleave at the hydrophobic amino acid residues Leu, Ile, Phe, Val, Ala, and Met (Arnold et al, 1996) with primary cleavage sites of Phe, Ile, Leu, and Val (Bark et al, 2001). The ability of thermolysin to digest PrP^{C} while leaving PrP^{Sc} intact results from the absence of thermolysin cleavage sites within the N-terminal region of PrP. While the first cleavage site for thermolysin is located at amino acid residue Met112 the first primary cleavage

site is located at residue Val115, and these residues are located with the protease resistant core region of PrP rendering them inaccessible in PrP^{Sc} but susceptible to digestion in PrP^{C}. PrP^{Sc} was found to possess considerable resistance to thermolysin digestion, with full length PrP^{Sc} remaining after 12 hours of digestion at 70°C with additions of 100µg/ml thermolysin per hour. It should be noted, though, that at these extended incubation periods some truncation of PrP^{Sc} was observed as evidenced by the loss of epitope to anti-PrP antibody AG4 in comparison to SAF32. As AG4 binds N-terminally (located at amino acid residues 31-51, with a secondary non-linear epitope at residues 151-167 which appears not to represent a significant epitope in SDS-denatured samples) to the SAF32 epitope (at amino acid residues 79-92) in PrP, a relative loss of AG4 epitope signal would indicate some N-terminal truncation of PrP^{Sc}.

Optimisation of thermolysin digestion conditions for ovine scrapie and bovine BSE brain homogenates indicated the generation of scrapie specific protein bands when analysed by Western blot using an antibody (SAF32) specific to the octapeptide repeat region of PrP. A comparison of three strain/host combinations (namely ovine scrapie, ovine BSE and bovine BSE) indicated that thermolysin digested ovine BSE PrP^{Sc} produced an intermediate banding pattern to ovine scrapie and bovine BSE PrP^{Sc} when detected with SAF32. However, the use of alternate anti-PrP antibody (P4) was better suited to strain discrimination in the same host species, and its use in conjunction with thermolysin led to the robust differentiation of ovine scrapie from ovine BSE using densitometry to identify scrapie-specific peptide bands within the caudal medulla region.

The absence of amino terminal truncation of PrP^{Sc} using thermolysin does raise an obvious potential drawback compared to PK in identifying that digestion has occurred. When analysed by Western blotting PK digestion of TSE positive brain homogenates generally results in both the persistence of a PrP^{Sc} signal and an obvious change in migration profile, with protease-resistant core PrP^{Sc} migrating with a lower apparent molecular weight compared to undigested PrP^{Sc} and PrP^{C}. This provides a direct in-process control for activity of the protease. In contrast, thermolysin digestion results in no obvious reduction in the overall migration profile of all PrP^{Sc} species under similar digestion conditions and therefore requires the use a healthy control sample to confirm that the digestion conditions selected resulted in the total clearance of PrP^{C}.

It should also be noted that upon digestion of CNS homogenates prepared from healthy animals that on occasion low levels of a PrP^{C} species of approximately 22kDa was observed following thermolysin digestion of CNS homogenate from healthy animals when detected with an antibody that binds to an epitope in the carboxy-terminal region of PrP (e.g. figure 3.9, panel B). This apparently protease-resistant PrP^{C} was not observed when using antibodies specific to the N-terminal region, suggesting that this species of PrP^{C} was amino-terminally truncated, and could be readily differentiated from PrP^{Sc} by their molecular weight profile as well as their lack of amino-terminal epitopes. Full-length thermolysin-resistant PrP^{C} was not detected however, and thermolysin-resistant PrP^{Sc} was present as glycosylated and unglycosylated full-length species with molecular weights ranging from 22kDa to 37kDa. Protease-resistant PrP^{C} species from healthy animals have previously been noted for PK digestions (Hope *et al*, 1999) and Pronase E digestions (D'Castro *et al*,

2010), and raises the possibility of misidentification of healthy animals as TSE positive since truncated PrP^{C} species migrate with a similar apparent molecular weight to protease-resistant core PrP^{Sc}. In contrast, apparently protease resistant PrP^{C} and PrP^{Sc} could readily be differentiated using thermolysin and antibodies specific to the N-terminal region of PrP.

The use of a protease which generates full-length PrP^{Sc} as opposed to amino-terminally truncated PrP^{Sc} also offers potential use in the area of protein diagnostics, as evidenced by the use of a sandwich ELISA within this work dependent on thermolysin-resistant PrP^{Sc} capture using an anti-PrP antibody specific to the N-terminal region of PrP, followed by detection of bound PrP^{Sc} using a carboxy-terminal specific anti-PrP antibody. A number of anti-PrP antibodies specific to the amino-terminal region have been generated and this region appears to present a significant number of the potential epitopes for the PrP protein, including repeated epitopes such as that described previously for SAF32 (Owen *et al.*, 2007). As such, the use of thermolysin allows for a significantly wider range of anti-PrP antibodies for PrP^{Sc} detection and quantification than does the use of proteinase K. The ability to select from a wider panel of antibodies covering the entirety of PrP^{Sc} as opposed to solely targeted to the protease-resistant core may result in more sensitive assays for PrP^{Sc} detection.

Leading on from the work presented in this study (and first published in 2007 as (Owen *et al.*, 2007)), the potential of thermolysin as a complementary tool to PK has subsequently been highlighted by Cronier and co-workers (Cronier et al, 2008) as a means to identify the presence of both PK-sensitive and PK-resistant isoforms of

disease related PrP. Evidence is mounting that PK-sensitive pathological isoforms of PrP may play a role in prion disease, as evidence by the absence of PK-resistant PrP^{Sc} in the presence of prion infectivity (Barron *et al.*, 2007) and scrapie strains such as Nor98 which display low levels of PK-resistant PrP under conditions classically used to identify PK-resistant PrP in CNS tissue (Benestad et al, 2008). Furthermore, Cronier and colleagues were also able to detect full-length thermolysin-resistant PrP^{Sc} in both human vCJD CNS homogenate and brain homogenate from Sc237 infected hamster, establishing that thermolysin-resistant PrP^{Sc} is not limited to the ruminant host/prion strain combinations first described by Owen and colleagues ((Owen *et al.*, 2007), and this work). Thermolysin-resistant PrP was further found to represent up to 90% of the total PrP^{Sc} in vCJD infected human brain, compared to approximately 10-20% of the total PrP^{Sc} being PK-resistant. Thermolysin can therefore be used as a complementary tool to PK in the study of PK-sensitive disease-associated isoforms of PrP. The novel use of thermolysin in TSE diagnostics has also led to the investigation into the potential use of other proteases for the study of PrP^{Sc}. Like thermolysin, digestion of CNS homogenates with 100µg/ml pronase E (synonyms: protease type XIV, actinase E) has also been found to result in the production of full length PrP^{Sc} from RML prion infected mouse brain (D'Castro *et al*, 2010). This result appears in contrast to results obtained in this work with scrapie infected ovine and BSE infected bovine brain homogenates, whereby truncation of PrP^{Sc} leading to the generation of PrP^{Sc} similar to $PrP^{27\text{-}30}$ was observed (figure 3.2 and table 3.1). It should be noted, however, that the concentration of 320µg/ml pronase E used here was 3.2 fold greater than used by D'Castro and co-workers to generate full length RML mouse PrP^{Sc}. D'Castro did note that the use of elevated concentrations of protease type XIV (1mg/ml) resulted in a

loss of PrP^{Sc} signal and a transition from full length to truncated PrP^{Sc}. A similar effect has been observed upon the extended incubation of RML infected mouse brain homogenate with thermolysin by Cronier (Cronier *et al*, 2008) and the occasional truncation of PrP^{Sc} in TSE infected ruminant brain homogenates observed using thermolysin under standard conditions (e.g. figure 3.11). Therefore the concentration of protease and incubation conditions used are of considerable importance not only for the clearance of PrP^{C}, but also the generation of full length PrP^{Sc} and the maintenance of prion infectivity, and should be determined empirically for each protease and prion strain/host combination.

As the use of thermolysin and latterly pronase E has demonstrated, there is considerable interest in the novel use of a range of proteases for the study of the prion protein. The established use of proteinase K has previously limited molecular and structural studies of PrP^{Sc} species to a small PK-resistant subset, which may at best represent only 20-25% of the total of disease associated PrP within a tissue sample. Furthermore, the PrP^{Sc} remaining after PK digestion is truncated and not necessarily representative of that found *in vivo*. Future investigations into thermolysin-resistant, pronase-resistant, and other novel protease-resistant PrP species (which may represent the majority of disease associated PrP species within an animal) warrants further investigation. Investigations into the contribution of the N-terminal domain to disease pathogenesis may also be possible with thermolysin digested PrP^{Sc}, as well as improved TSE diagnostics and PrP^{Sc} isolation procedures via N-terminal capture are further areas where the use of thermolysin digestion of PrP^{Sc}-containing CNS homogenates may be appropriate.

Finally, although the functional role and physicochemical properties of the amino-terminal region of PrP^{C} have been previously documented (for example (Nunziante *et al.*, 2003)), the study of this region from the disease isoform has been more limited, notably because almost all analyses of PrP^{Sc} have involved the truncation of PrP^{Sc} with PK. Work by Yam and co-workers into the proteolytic digestion of PrP^{Sc} with trypsin (resulting in the production of amino-terminally truncated PrP^{Sc} with the octapeptide repeat region intact), has indicated that the N-terminal region undergoes conformational changes upon conversion from PrP^{C} to PrP^{Sc} (Yam et al, 2004), since epitope exposure studies resulted in an increase in antibody binding by octapeptide repeat targeted antibodies upon denaturation of PrP^{Sc}. As Yam and colleagues found that the use of trypsin resulted in truncation of the first 48 amino acids of PrP^{Sc}, the use of thermolysin and the analysis of amino-terminally intact PrP^{Sc} may have yielded results more closely related to the physiological conformations of PrP^{Sc}.

6.2 The use of a thermolysin for the strain typing of ruminant TSEs.

The elucidation of prion strains within the UK ruminant population is of fundamental importance given the public health risk posed by prion zoonoses such as bovine spongiform encephalopathy. To this end, identification of emergent TSE strains requires rapid and robust assays capable of readily distinguishing prion strains. Many new prion diseases have so far been identified arising as a result of the BSE epidemic in UK cattle (Sigurdson and Miller, 2003), and, given the promiscuous nature of BSE with regards to interspecies transmission the rapid identification of emergent TSE strains potentially capable of infecting humans would appear advisable.

A number of different methods exist for the classification of prion strains, the "gold standard" being prion strain typing using mice bioassay by the analysis of neuronal lesion profiles. This method provided strong evidence that vCJD in humans was caused by the same prion strain as BSE in cattle (Bruce *et al.*, 1997) and has been used to reliably differentiate scrapie strains (Fraser and Dickinson, 1973). The main drawbacks to this method are the relatively long time periods required before a result can be obtained and species barrier effects. Similarly, immunohistochemical methods have been used for the identification of prion strains based on the detection and distribution of PrP^{Sc} in the brain and in lymphoid tissues. In the case of vCJD and BSE, the similarity of "florid plaques" in macaques infected with BSE further supported the hypothesis of a link between vCJD and BSE (Lasmezas *et al.*, 1996).

In contrast to the above two methods, modern molecular methods of strain typing typically rely on the biochemical characteristics of PrP^{Sc} and its partial resistance to proteolysis, with analysis of PrP^{Sc} isotypes resolved by polyacrylamide gel electrophoresis and immunological detection using PrP specific antibodies. Such methods have been used in conjunction with glycoform profiling for the classification of CJD cases (Collinge *et al.*, 1996) and to support the hypothesis of a link between vCJD and BSE (Hill *et al.*, 1997). This method is somewhat hampered, however, by the requirement to reliably identify very subtle differences in migration profiles of PK digested PrP^{Sc} in order to successfully categorise strains. This inherent difficulty led to the decision to limit investigation in to two alternate proteases (bromelain and protease XIV) identified in this work as capable of discriminating between two TSEs in the same host species.

A further method of molecular strain typing developed by Stack and co-workers relied upon the differential binding of antibodies to proteinase K digested samples as well as glycoform profiling and differential migration to discriminate between ovine scrapie, experimentally infected ovine BSE, and the "BSE-like" scrapie strain CH1641 (Stack *et al.*, 2002). In comparison to this and other previously published strain typing assays the thermolysin strain-typing assay does not rely on very subtle differences in the molecular weights of PrP^{Sc} bands generated by PK digestion of BSE and scrapie samples. Additionally, it does not require the multiple analyses of samples as is required for glycoform ratio determination, nor does it require the analysis of the same samples on multiple gels as is necessary for the assessment of quantitative differences in the binding of anti-PrP antibodies (Stack *et al.*, 2002).

This work describes the novel use of repeated additions of relatively high concentrations of the protease thermolysin to produce Western blot PrP^{Sc} profiles capable of distinguishing two prion strains within the same host species (namely classical ovine scrapie covering a range of prion genotypes and experimental ovine BSE of genotype ARQ/ARQ, as described in table 2.1) when detected using antibodies targeted to the N-terminal region of PrP. A comparison of 15 caudal medulla samples isolated from ovine scrapie animals with 4 BSE infected sheep yielded in every case scrapie- or BSE-specific PrP^{Sc} fragment profiles using the PrP specific antibody P4. Ovine scrapie samples yielded significant P4-reactive PrP peptides with apparent molecular weights of approximately 38, 28, 23, and 19kDa, while BSE samples contained significant amounts of PrP^{Sc} species between 36 and 27kDa. There appeared

to be no link between the PrP genotype or sheep breed and the generation of molecular profiles in scrapie samples.

A further analysis of caudal medulla, cerebellum, and spinal cord from 11 ovine scrapie cases, and caudal medulla and cerebellum from a further 3 isolates indicated all spinal cord and caudal medulla samples generated typical scrapie PrP^{Sc} profiles. In contrast, the cerebellum samples generated two distinct deposition patterns of thermolysin-resistant PrP^{Sc}: 12 isolates produced typical scrapie PrP^{Sc} profiles following the addition of thermolysin sequentially for 8 hours, while 2 isolates lacked any detectable PrP^{Sc} after only 1 hour of thermolysin digestion. It is plausible that the differences observed when using the thermolysin assay in relation to these two groupings represent different, naturally occurring, scrapie strains. It has been previously observed that different PrP^{Sc} strains target different brain regions (DeArmond *et al.*, 1993), however the above interpretation is subject to the caveat that it is unknown what effect factors such as route of infection, dose of infectivity, or age of exposure may have on the deposition of PrP^{Sc} within the host brain.

The analysis, using the thermolysin strain-typing assay, of brain tissue from animals experimentally infected with two well characterised scrapie strains (CH1641 and SSBP/1) indicated two very different patterns of PrP^{Sc} deposition within the cerebellum which are not believed to result from the different routes of infection used to produce these experimentally infected animals. SSBP/1 gave scrapie-associated bands even after 8 repeat digestions with thermolysin whereas the CH1641 failed to produce any scrapie-specific bands after as little as 1 hour digestion. This differential deposition of PrP^{Sc} with the cerebellum appears to support the hypothesis that region-

specific deposition may be linked to TSE strain. In contrast, a comparison of PrP^{Sc} profiles following thermolysin digestion of prion strains CH1641 and SSBP/1 passaged through ovinised mice by Nicot and Baron did not identify an absence of thermolysin-resistant PrP^{Sc} in CH1641 compared to SSBP/1. Whether this was due to the use of whole mouse brain homogenates precluding an analysis of the regional deposition of PrP^{Sc}, or an effect arising as a result of using a mouse model, is unknown (Nicot and Baron, 2010). The authors speculated that the differences between the two studies may have been due to differences in PrP^{Sc} levels within ovine cerebellum compared to the ovinised transgenic mouse model (TgOvPrP4), however levels of PK-resistant PrP^{Sc} in CH1641 cerebellum used in this study were unremarkable compared to, for example, SSBP/1 cerebellum PrP^{Sc} levels.

It has been previously suggested for CJD that the exact profile of PrP^{Sc} peptides, and not merely the protease resistant core, correlates with neuropathological phenotypes (Jimenez-Huete et al, 1998). To date, two major PrP fragments have been described within both human and murine tissues: the C1 fragment of PrP generated in vivo as a result of disintegrin-mediated cleavage (Chen *et al.*, 1995), and the C2 fragment which is present at very low levels in healthy individuals but accumulates during TSE disease and is due to either calpain-dependent cleavage of PrP (Chen *et al.*, 1995; Pan *et al.*, 2005) or the action of acidic hydrolases (Dron *et al.*, 2010). The presence of the C2 band within ovine samples is described within this work, and it appeared that C2 generation was strain specific within the samples analysed for this work; C2 was readily detected in numerous natural scrapie cases but absent in experimental BSE. C2 was also readily detected in ovine scrapie strain SSBP/1 but not in ovine scrapie strain

CH1641, indicating that the presence of C2 cannot be classified as a marker of TSE infection, rather it appeared to be indicative of the presence of a SSBP/1-like strain or strains within the UK flock. No C2 fragment was detected in either healthy ovine or healthy bovine homogenates.

Using a combination of the neuroanatomical deposition of thermolysin-resistant PrP^{Sc} and the presence of C2 allowed the classification of field scrapie cases in to four distinct groups. The majority of field cases demonstrated thermolysin-resistant PrP^{Sc} and C2 fragment within all CNS regions tested. A second group was characterised by thermolysin-resistant PrP^{Sc} within the CNS but an absence of cerebellum C2 (animals 0455/03 and 0226/03). A single isolate (animal 0456/03) demonstrated an absence of thermolysin-resistant PrP^{Sc} and C2 fragment in the cerebellum, and finally a single isolate (animal 0284/97) was characterised by an absence of thermolysin-resistant PrP^{Sc} in the cerebellum and an absence of C2 in all CNS regions. It is possible that these groupings therefore reflect the heterogeneity in scrapie strains associated with the occurrence of scrapie in the UK flock.

The limited availability of caudal medulla tissue from all experimental prion strains, which was the preferred CNS tissue for strain typing analysis using thermolysin, limited the number of strain comparisons that could be made. Ideally caudal medulla and cerebellum from a wider range of classical and atypical scrapie strains would have been available, along with spinal cord tissue from ovine animals experimentally infected with BSE from which caudal medulla and cerebellum was made available. The demand for tissues from scrapie field cases and animals experimentally infected with BSE is understandably great, however it is believed the lack of availability of these

tissues did not limit the conclusions that can be drawn from the samples available; namely that a method for the differentiation of TSE strains within a single host species utilising the novel use of the protease thermolysin has been developed, and the apparent strain-related distribution pattern of both PrP^{Sc} and PrP^{Sc} endogenous cleavage products within separate CNS from natural scrapie isolates within the UK flock have been detected.

In conclusion, a novel strain typing assay has been developed using the protease thermolysin which, under optimised digestion conditions, was capable of distinguishing between ovine BSE and classical ovine scrapie based on the densitometry profile of thermolysin digested caudal medulla homogenates. A further difference was also observed in the occurrence of thermolysin-resistant PrP^{Sc} within cerebellum homogenates of CH1641 and SSBP/1 and data indicated that while SSBP/1 produced a PrP^{Sc} profile similar to the majority of cerebellum samples from scrapie field cases, CH1641 cerebellum was notable for an absence of thermolysin-resistant PrP^{Sc}. An absence of protease-resistant PrP^{Sc} from cerebellum tissue was also noted for two field cases from which thermolysin-resistant PrP^{Sc} was detected in the caudal medulla, and may be indicative of strain characteristics. The presence of C2 fragments was also observed in ruminants for the first time and appeared to be strain specific, with ovine BSE and certain field scrapie cases notable for an absence of C2. Analysis of these apparent strain characteristics using other strain typing assays and/or lesion profiling could be used to complement the data presented here and provide a greater understanding into the nature of TSE strains.

The possibility has been raised that atypical cases of BSE and scrapie could also be classified using the thermolysin assay with the potential for identification and characterisation of novel TSE strains in other species. Further analysis of archived tissues from a wider range of scrapie cases and other TSEs from various host species is therefore believed warranted, particularly in view of recent work suggesting that multiple scrapie strains may occur in a single host (Yokoyama *et al.*, 2010). It seems probable that interest in the use of thermolysin and other novel proteases will continue for the identification of PrP species both as a method of strain characterisation, and for the analysis of disease-related PK-sensitive isoforms of PrP^{Sc}.

6.3. The persistence of PrP^{Sc} in soil.

The persistence of the TSE agent within the environment is an important issue which requires further study given the considerable stability that PrP^{Sc} seems to display and the obvious presence of environmental reservoirs of infectivity for scrapie and CWD. Persistence of CWD within enclosures previously used to house CWD-affected animals has previously been demonstrated (Miller *et al.*, 2004), and while evidence suggests that CWD is not transmissible to transgenic mice overexpressing human PrP (Sandberg *et al.*, 2010) or wild-type macaques (Race *et al.*, 2009), the UK BSE epidemic and subsequent vCJD cases has highlighted the potential risk posed to humans from dietary exposure to CWD. Low levels of infectivity have been detected in water and bedding (Mathiason *et al.*, 2009), urine and saliva (Haley *et al.*, 2009b), faeces (Haley *et al.*, 2009a; Tamguney *et al.*, 2009), blood (Mathiason *et al.*, 2009), and antler velvet (Angers *et al.*, 2009) from CWD affected animals as well as blood from BSE- and scrapie-infected sheep (Hunter *et al.*, 2002), and as a whole this data suggests several

potential sources for environmental prion contamination. Prions are known to bind to soil and soil minerals (Johnson *et al.*, 2007; Saunders *et al.*, 2009a; Seidel *et al.*, 2007) indicating a likely reservoir of infectivity, therefore an investigation into the prion retention properties of UK soils of two TSEs relevant to the UK, namely ovine scrapie and bovine BSE was carried out in this work.

In addition to CWD, horizontal transmission of scrape has been shown to occur under farm conditions (Hoinville, 1996), with PrP^{Sc} having been detected on a number of surfaces from which environmental transmission could potentially occur (Maddison *et al.*, 2010a). There is circumstantial evidence of scrapie infectivity remaining within an Icelandic farm building for a period approaching two decades (Georgsson *et al.*, 2006). Although an isolated report such as this over such an extended period must be accepted with some caution, the stability of PrP^{Sc} to thermal, chemical and proteolytic degradation suggests that long-term environmental persistence caused by shedding of PrP^{Sc} from infected animals within the environment may be a hazard worthy of recognition given the risk to human health of BSE and the centuries-long presence of scrapie within the UK flock. Selective breeding has been suggested as a means of reducing the incidence of scrapie (Elsen *et al.*, 1999), and considerable effort via the National Scrapie Plan has been taken to breed out scrapie from the UK flock through the selection of genotypes more resistant to scrapie infection. However, this does not preclude the emergence of scrapie strains more capable of propagation within resistant genotypes (or the continued persistence of sub-clinical infection within these animals), or as a result of the importation of susceptible animals.

The susceptibility of goats to classical scrapie is also worthy of mention, as no breeding program is believed to be in place to select for resistant genotypes in goats. Genetic resistance to scrapie in goats is not as well characterised as within sheep, and although some polymorphisms have been identified which confer increased incubation times following scrapie exposure (Vaccari *et al*, 2006) to date no polymorphisms have been characterised which confer absolute resistance to classical scrapie.

Given the likelihood that soils represent an environmental reservoir for scrapie and CWD infectivity, the apparent increase in infectivity observed by Johnson and co-workers when orally dosing mice with soil-bound as opposed to un-bound PrP^{Sc} may indicate that even relatively small amounts of PrP^{Sc} remaining within the upper soil layers would still be capable of generating new cycles of infectivity (Johnson *et al.*, 2007). The tight-coupling of PrP^{Sc} to soil particles even in the presence of treatment with high levels of detergents at elevated temperatures observed both in this work and by others analysing the binding and desorption of unglycosylated recombinant PrP from mica (Vasina et al, 2005) suggests that migration of PrP^{Sc} to the subsoil layers may be a relatively slow process. This would further increase the possibility of TSE infectivity remaining in a host-accessible region of the environment.

In the work presented in this thesis the sorption and elution of two PrP^{Sc} strains to different types of UK soil was investigated, and while PrP^{Sc} bound rapidly and completely to all soils tested elution was found to be dependent on soil type with sandy soil giving the highest levels of PrP^{Sc} elution. Intermediate levels of recovery were observed from clay soil, with recovery lowest from organic-rich clay soil. This

data was in agreement with previous studies using rodent adapted TSEs which indicated that binding of PrP^{Sc} to soil occurred rapidly and completely (Johnson *et al.*, 2006) and that harsh conditions were required to elute PrP^{Sc}. In this thesis the treatment of soil bound PrP^{Sc} with both protease and boiling 10% SDS was found to elute only the minority of PrP^{Sc} from all the soils tested, and little or no PrP^{Sc} was found in soil washes. In agreement with work by Seidel on the persistence of Hamster adapted 263K scrapie prions incubated with soil, both ovine scrapie PrP^{Sc} and bovine PrP^{Sc} were found to persist on soil for extended periods (Seidel *et al.*, 2007), and, given the remarkable stability that PrP^{Sc} displays under proteolytic conditions (this thesis and (Owen *et al.*, 2007)) it seems probable that the low recovery of PrP^{Sc} observed is due largely to irreversible binding as opposed to proteolysis.

As previously noted, the sand or clay content of soil appears to be the predominant factor in determining PrP^{Sc} elution, with the presence of organic material such as found in Cambs2 soil exacerbating the low recoverability. This data would confirm the conclusions of Cooke and co-workers that murine prions were more readily eluted from sand-rich soils compared to clay-rich soils (Cooke *et al.*, 2007). Furthermore, the highest retention of prion within soil with both high clay and organic matter contents may indicate an accumulative or synergistic effect of humic substances and clay minerals in the high affinity interactions with PrP^{Sc}.

It was determined that not only did soil type affect the overall levels of PrP^{Sc} elution, but also the level of N-terminal truncation that was detected upon elution. In contrast to the relatively high levels of full-length PrP^{Sc} recovered from sandy soils, clay rich soils resulted in the truncation of PrP^{Sc}, with the silt-rich soil Hereford yielding a

mixture of both full length and truncated PrP^{Sc}. While it was not known whether the truncation occurred during the binding or desorption phases of PrP^{Sc} interaction with clay-rich soils, this data is in agreement with previous studies which have indicated an N-terminal truncation of PrP^{Sc} occurs upon interaction with clay soils (Cooke *et al.*, 2007; Davies and Brown, 2009; Johnson *et al.*, 2006). PrP^{Sc} is believed to bind to clay soil particles initially via the unstructured N-terminal region, and although binding studies with PK digested hamster-adapted TME PrP^{Sc} (and hence lacking an N-terminal region) indicate the N-terminal region is not a prerequisite for PrP-clay particle interaction (Jacobson *et al.*, 2010), it does appear to have a role in prion interaction with both clay- and sand-rich soils with the presence of the N-terminal domain suggested to facilitate enhanced sorption to clay soils and reduced sorption to sandy soils (Saunders *et al.*, 2009a). Notably, binding of PrP^{Sc} to montmorillonite, an aluminium phyllosilcate clay mineral, was found by Johnson and co-workers to occur within the order of 1 minute of co-incubation, with N-terminal cleavage of PrP^{Sc} occuring within 15 minutes. This may suggest that binding of PrP^{Sc} to clay minerals in a field environment could also occur extremely rapidly and may result in PrP^{Sc} binding at or near the site of deposition. Notably, temperature had an effect of the truncation of PrP^{Sc} following incubation with Notts1 soil over an 11 day period when performed in a batch-binding experiment. Incubation of ovine scrapie brain homogenate at 25°C with this soil resulted in a reduction of full-length PrP^{Sc} compared to 4°C, while levels of total PrP^{Sc} remained similar at both temperatures. Our previous observations had demonstrated that ovine scrapie PrP^{Sc} underwent relatively little N-terminal truncation on Notts1 soils compared to clay- and silt-rich soils such as Cambs3 and Hereford following 24 hours incubation at 16-20°C. This would suggest that N-terminal

truncation is affected by both soil type and temperature. Data by Johnson and co-workers has described an absence of N-terminal truncation of hamster passaged TME PrP^{Sc} to quartz sand following 2 hours binding (Johnson *et al.*, 2006), while the sandy soil Notts1 described here also contains a proportion of silt and clay particles representing approximately one third of the total inorganic fraction. In the absence of a more detailed characterisation of the components composing this smaller fraction of Notts1 soil it is likely that the heterogeneity of Notts1 soil compared to quartz sand may have a role in the amino terminal truncation observed in this work.

The persistence of misfolded prion protein, namely ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc}, was monitored on 6 UK soils over a period of 18 months. Recovery of PrP^{Sc} was found to be dependent on soil type, and recoverable levels of PrP^{Sc} declined over time. Both ovine scrapie and bovine BSE PrP^{Sc} were readily recovered from soils Notts1 and Notts2 after a total of 18 months co-incubation. Elution of PrP^{Sc} from soil rich in organic material was the least efficient, with recoveries in the order of 5% after 24 hours and recovery below the level of detection within a 1 month period. Despite this, sufficient PrP^{Sc} was eluted from this soil after 18 months to act as a template for PMCA, suggesting that PrP^{Sc} could remain in the environment below the level of detection of traditional Western blotting techniques and yet act as a potential source of infectivity. PMCA has been demonstrated to be able to detect PrP^{Sc} on and in a range of surfaces and materials from a farm facility used for the husbandry of ruminants infected for TSE research 20 days after the removal of sources of infectivity (Maddison *et al.*, 2010a), as well as from soil after 21 months co-incubation (Seidel *et al.*, 2007). While the data from Maddison was over a relatively short period of time

the possibility exists that long term exposure to trace levels of TSE pathogenic material will occur on farms where TSE diseases have previously been confirmed and remedial action taken. Data presented here indicated that incubation of ovine scrapie brain homogenate for 6 months at 16-20°C in the absence of soil resulted in little or no reduction in either total PrP, or PrP^{Sc} detected by Western blotting following PK digestion. The absence of truncation of PrP over 6 months in the absence of soil therefore indicated that differences in PrP^{Sc} persistence during incubation with soil as described in this work arose as a result of soil effects and not endogenous protease activity. While this is not in agreement with data generated by Saunders which indicated that rodent passaged TME, mouse passaged CWD and elk CWD brain homogenates underwent proteolysis of PrP^{Sc} upon incubation at 22°C (Saunders *et al.*, 2008), Saunders also observed that cervid CWD was not subject to proteolysis from endogenous brain proteases at 22°C (Saunders *et al.*, 2009a). The reason for the discrepancies between the work presented here and Saunders findings is not known, however it is possible that both strain differences and difference in methodology were responsible for the different observations.

There is some evidence to suggest that manganese is involved in increasing persistence of recombinant PrP when bound to a montmorillonite-manganese matrix as opposed to montmorillonite alone, and that manganese sulphate can enhance prion infectivity in a cell-based assay described by Davies (Davies and Brown, 2009). In contrast, Russo and colleagues described that manganese oxide can increase the degradation of purified hamster-passaged TME PrP^{Sc}, particularly at low pH (Russo *et al.*, 2009). In the work described here, no correlation was observed between

manganese levels or pH and the persistence of ovine scrapie or bovine BSE PrP^{Sc}. This would suggest that in complex, heterogeneous matrices such as the soils investigated in the course of this work the role of manganese may be relatively minor compared to other factors affecting PrP^{Sc} persistence such as the organic or clay content.

Following spiking of soil columns with brain homogenate the deposition of PrP^{Sc} within the soil columns was analysed by separating columns into thirds and analysing PrP^{Sc} within each section. While no migration of PrP^{Sc} through the soil column was subsequently observed over time, the initial deposition of PrP^{Sc} within the soil column was dependent on soil type. For Notts1 and cambs3 soil PrP^{Sc} was predominantly in the top one third of columns, for Notts2 soil PrP^{Sc} was found within the top two thirds of columns, while for Cambs1, Cambs2 and Hereford soils PrP^{Sc} was found within the entire column depth. This trend was unaffected by prion strain and did not display an obvious correlation with soil properties such as sand, clay or moisture content. This data suggests that PrP^{Sc} binds to soil avidly shortly after initial contact and that once bound PrP^{Sc} is not released over time, since analysis of PrP^{Sc} deposition 6 and 18 months after spiking demonstrated similar deposition patterns (albeit with a reduced level of eluted PrP^{Sc}). This data is in agreement with previously published work indicating that little or no migration of hamster adapted 263K scrapie PrP^{Sc} occurs after initial deposition (Seidel *et al.*, 2007). While this would suggest that environmental contamination with TSE agents could be expected to remain within the upper soil layers for extended periods and hence remain accessible to grazing animals, it could also suggest that infectivity arising from, for example, the burial of infected carcasses will have little potential for movement away from the burial site and is

unlikely to present a further risk in terms of contamination of groundwater. Similarly, weathering effects such as heavy rain may only have a very limited effect on the migration of infectivity into the subsoil, thereby maintaining bioavailability.

Raising and lowering the pH of Notts1 soil also seemed to have no effect on PrP^{Sc} migration over time or the persistence of PrP^{Sc}, although soil pH did appear to affect PrP^{Sc} deposition upon initial application. At lower pH values PrP^{Sc} was found to bind rapidly to the upper third of the column, while at higher pH values PrP^{Sc} was observed to be carried further into the soil column. Application of brain homogenate suspensions to Notts1 soil columns typically wetted the entire column depth within 15 minutes (data not shown), suggesting that complete binding of PrP^{Sc} was occurring to the upper regions of the column within this period at acidic pH. Furthermore, an investigation into the recovery of full-length ovine scrapie PrP^{Sc} from Notts1 soil following 1 and 4 hour incubation periods at 4 defined pH values showed that there was less recoverable PrP^{Sc} from soil samples at elevated pH, with a corresponding increase in PrP^{Sc} in the supernatant fraction. This would suggest that the binding of PrP^{Sc} to soil components is likely to be affected by pH and may account for the greater penetration of prion seen in the study at higher soil pH values, and is in agreement with work by Ma and colleagues which suggested that maximal binding of PrP^{Sc} aggregates to quartz sand occurs at the isoelectric point of the prion aggregates (pH4.6) and is reduced at basic pH values (Ma *et al.*, 2007)

Ovine scrapie and bovine BSE PrP^{Sc} were found to bind differentially to soil size fractions dependent on both soil type and TSE strain. For the majority of soils tested PrP^{Sc} bound predominantly to soil particles greater than 63µm, or equally between

soil fractions. For a single soil, namely Notts2, both ovine scrapie PrP^{Sc} and bovine BSE PrP^{Sc} was found to bind predominantly to the fraction containing soil particles less than 63µm in size which constituted approximately 14% of the soil total. The reason for this differential binding preference is unknown, and did not appear to be dependent on the composition of the inorganic fraction of the soil.

In addition to data that identified that soil type has a considerable effect on ovine scrapie and bovine BSE PrP^{Sc} persistence, this work also identified the temperature at which PrP^{Sc} and soil are co-incubated as a factor influencing PrP^{Sc} persistence. The persistence of PrP^{Sc} on Notts1 soil was found to be inversely correlated to temperature when analysed by both Western blot and by ELISAs which measured full-length PrP^{Sc} and total PrP^{Sc}, i.e. as temperature decreased PrP^{Sc} persistence increased. PrP^{Sc} levels following extended incubation on soil at 4°C were typically two fold higher than those observed at 25°C, and remained relatively constant up to the 566 day time point. This data also confirmed the absence of strain and/or host effects with regards to temperature as indicated by qualitative Western blot. Under field conditions this may mean that the retention of PrP^{Sc} in soil occurs for a potentially longer time in cooler climates than in warmer ones.

Little data has been published on the persistence of proteins on soil as a result of differences in soil moisture content. Yao-yu and co-workers investigated the persistence of *Bacillus thuringenisis* Cry1Ab protein on three soils maintained at 50%, 70% and 100% of water-holding capacity and observed a decrease in degradation linked to increasing moisture content on two soils and an increase in degradation linked to moisture content with a third soil (Yao-yu *et al.*, 2007). Therefore the

influence of soil moisture persistence is a complex phenomenon dependent not only on the moisture content of the soil but also the soil type. In this work an analysis of the recoverability of PrP^{Sc} in soil columns maintained at different field moisture contents indicated an increase in elution efficiency of ovine scrapie PrP^{Sc} linked to decreasing moisture content from Notts1 soil following 24 hours incubation. This data was consistent for FMC and -60% FMC soil up to 188 days

6.4. General Discussion and future work

Overall, this thesis presents a number of novel findings. Firstly, the novel use of a protease capable of generating full length PrP^{Sc} free from PrP^{C} contamination. Secondly, the use of thermolysin for a novel strain typing assay capable of distinguishing between different prion strains in the same host species, and thirdly the first data on the persistence of PrP^{Sc} on a range of UK soils and a single UK soil analysed under varying environmental conditions.

The novel use of thermolysin for the diagnosis of prion diseases has indicated that proteases complementary to PK can be used for studying the PrP^{Sc} molecule. While PK has traditionally been used for the clearance of PrP^{C} and identification of protease resistant PrP^{Sc}, its use results not only in the amino terminal truncation of PrP^{Sc} but also the digestion of disease-associated, PK-sensitive forms of PrP^{Sc}. Growing evidence suggests this fraction of PrP^{Sc} may represent a considerable portion of total PrP^{Sc}. The use of thermolysin may in future therefore not only permit the analysis and characterisation of full-length forms of PrP^{Sc} but also allow the investigation into the infectivity of that fraction of PrP^{Sc} labile to PK degradation.

This work presented data for a novel strain typing assay that suggests the neuroanatomical deposition of thermolysin-resistant PrP^{Sc} and C2 fragments may be linked to TSE strain. In order to fully characterise the observed differences experimental passage could be performed, firstly to determine whether these differences in pathology are maintained under experimental passage, and secondly to determine whether the molecular traits identified in this work are transmissible. This would go some way to validating the data presented here concerning the use of thermolysin-resistant PrP^{Sc} and C2 fragment profiles as markers of classical scrapie strains within the natural host.

The current study concentrated on the persistence and binding of ruminant prions to a range of soil types indicated that prions bind strongly and rapidly to multiple soil types and show considerable persistence under typical environmental conditions. Limited migration of prions was observed after application to soil, suggesting that prions excreted or secreted into the environment will probably be retained at or near the soil surface and remain accessible to grazing animals. This in turn would tend to facilitate the repeat occurrence of disease in locations where prion infection has previously been identified. Since variations in the interaction between prion strains and soil were detected this would suggest that subtle difference in conformation may influence the interaction between soil and PrP^{Sc}. Therefore future work should include studies into the interaction with soils of naturally occurring, environmentally relevant prions such as those excreted/secreted in urine, saliva, faeces and milk.

Factors such as the migration of PrP^{Sc} over time, the distribution of PrP^{Sc} in fractions based on soil particle size, the effect of defined pH, temperature, and moisture

content were analysed. Temperature was found to have a noticeable effect on the persistence of PrP^{Sc} on soil, and, while this work only monitored the fraction of PrP^{Sc} that could be eluted, future work could concentrate on the analysis of on-soil PrP^{Sc} levels or semi-quantitative PMCA to determine total PrP^{Sc} levels.

The novel use of the protease thermolysin capable of generating full-length PrP^{Sc} in the absence of contaminating PrP^{C} has led other workers to investigate not only thermolysin-resistant, PK-sensitive isoforms of PrP^{Sc} but also renewed interest in investigating other proteases, such as pronase E, as an alternative to proteinase K. Further work in this area may reveal more information on the infectivity of disease-associated forms of PrP^{Sc} which are sensitive to treatment with PK and may represent the majority of PrP^{Sc} within an animal. The isolation of full length PrP^{Sc} may also allow the development of novel isolation and detection methods relying on the use of PrP^{Sc} with an intact N-terminus.

Future analysis into the persistence of PrP^{Sc} within the environment may concentrate on the use of techniques such as PMCA to detect levels of PrP^{Sc} below the levels detectable using more classical methods such as ELISA and Western blot, and the direct detection of PrP^{Sc} on soil i.e. in the absence of a relatively inefficient elution step.

Given the observed persistence of PrP^{Sc} in UK soils observed in the work presented in this thesis a scrapie outbreak in the field should be considered an event requiring long term remedial action; either through culling with restocking using sheep genetically predisposed to scrapie resistance, via the removal and disposal of all potentially contaminated soil or buildings, or through cessation of sheep husbandry on the site.

REFERENCES

Andreoletti, O., Berthon, P., Marc, D., Sarradin, P., Grosclaude, J., van Keulen, L., Schelcher, F., Elsen, J.M., Lantier, F. Early accumulation of PrP(Sc) in gut-associated lymphoid and nervous tissues of susceptible sheep from a Romanov flock with natural scrapie. *J Gen Virol.* 2000. **81**, 3115-3126.

Angers, R.C., Seward, T.S., Napier, D., Green, M., Hoover, E., Spraker, T., O'Rourke, K., Balachandran, A., Telling, G.C. Chronic wasting disease prions in elk antler velvet. *Emerg Infect Dis.* 2009. **15**, 696-703.

Arnold, U., Rucknagel, K.P., Schierhorn, A., Ulbrich-Hofmann, R. Thermal unfolding and proteolytic susceptibility of ribonuclease A. *Eur J Biochem.* 1996. **237**, 862-869.

Axelrad, J. An autoimmune response causes transmissible spongiform encephalopathies. *Med Hypotheses.* 1998. **50**, 259-264.

Baron, T., Bencsik, A., Biacabe, A.G., Morignat, E., Bessen, R.A. Phenotypic similarity of transmissible mink encephalopathy in cattle and L-type bovine spongiform encephalopathy in a mouse model. *Emerg Infect Dis.* 2007. **13**, 1887-1894.

Baron, T., Bencsik, A., Morignat, E. Prions of ruminants show distinct splenotropisms in an ovine transgenic mouse model. *PLoS One.* 2010. **5**, e10310.

Baron, T.G., Madec, J.Y., Calavas, D. Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. *J Clin Microbiol.* 1999. **37**, 3701-3704.

Barron, R.M., Campbell, S.L., King, D., Bellon, A., Chapman, K.E., Williamson, R.A., Manson, J.C. High titers of transmissible spongiform encephalopathy infectivity associated with extremely low levels of PrPSc in vivo. *J Biol Chem.* 2007. **282**, 35878-35886.

Bastian, F.O. Spiroplasma as a candidate agent for the transmissible spongiform encephalopathies. *J Neuropathol Exp Neurol.* 2005. **64**, 833-838.

Bastian, F.O., Sanders, D.E., Forbes, W.A., Hagius, S.D., Walker, J.V., Henk, W.G., Enright, F.M., Elzer, P.H. Spiroplasma spp. from transmissible spongiform encephalopathy brains or ticks induce spongiform encephalopathy in ruminants. *J Med Microbiol.* 2007. **56**, 1235-1242.

Baylis, M., Chihota, C., Stevenson, E., Goldmann, W., Smith, A., Sivam, K., Tongue, S., Gravenor, M.B. Risk of scrapie in British sheep of different prion protein genotype. *J Gen Virol.* 2004. **85**, 2735-2740.

Beck, K.E., Chaplin, M., Stack, M., Sallis, R.E., Simonini, S., Lockey, R., Spiropoulos, J. Lesion profiling at primary isolation in RIII mice is insufficient in distinguishing BSE from classical scrapie. *Brain Pathol.* 2010. **20**, 313-322.

Beck, M., Asenova, D., Dickson, G. Public administration, science and risk assessment: a case study of the U.K. Bovine Spongiform Encephalopathy crisis. *Public Administration Review.* 2005. **65**, 396-408.

Bellworthy, S.J., Hawkins, S.A., Green, R.B., Blamire, I., Dexter, G., Dexter, I., Lockey, R., Jeffrey, M., Ryder, S., Berthelin-Baker, C., Simmons, M.M. Tissue distribution of bovine spongiform encephalopathy infectivity in Romney sheep up to the onset of clinical disease after oral challenge. *Vet Rec.* 2005. **156**, 197-202.

Bencsik, A., Baron, T. Histopathological studies of "CH1641-like" scrapie sources versus classical scrapie and BSE transmitted to ovine transgenic mice (TgOvPrP4). *PLoS One.* 2011. **6**, e22105.

Benestad, S.L., Arsac, J.N., Goldmann, W., Noremark, M. Atypical/Nor98 scrapie: properties of the agent, genetics, and epidemiology. *Vet Res.* 2008. **39**, 19.

Beringue, V., Andreoletti, O., Le Dur, A., Essalmani, R., Vilotte, J.L., Lacroux, C., Reine, F., Herzog, L., Biacabe, A.G., Baron, T., Caramelli, M., Casalone, C., Laude, H. A bovine prion acquires an epidemic bovine spongiform encephalopathy strain-like phenotype on interspecies transmission. *J Neurosci.* 2007. **27**, 6965-6971.

Beringue, V., Bencsik, A., Le Dur, A., Reine, F., Lai, T.L., Chenais, N., Tilly, G., Biacabe, A.G., Baron, T., Vilotte, J.L., Laude, H. Isolation from cattle of a prion strain distinct from that causing bovine spongiform encephalopathy. *PLoS Pathog.* 2006. **2**, e112.

Beringue, V., Mallinson, G., Kaisar, M., Tayebi, M., Sattar, Z., Jackson, G., Anstee, D., Collinge, J., Hawke, S. Regional heterogeneity of cellular prion protein isoforms in the mouse brain. *Brain.* 2003. **126**, 2065-2073.

Bessen, R.A., Marsh, R.F. Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *J Virol.* 1994. **68**, 7859-7868.

Biacabe, A.G., Laplanche, J.L., Ryder, S., Baron, T. Distinct molecular phenotypes in bovine prion diseases. *EMBO Rep.* 2004. **5**, 110-115.

Brazier, M.W., Davies, P., Player, E., Marken, F., Viles, J.H., Brown, D.R. Manganese binding to the prion protein. *J Biol Chem.* 2008. **283**, 12831-12839.

Brown, D.R. BSE did not cause variant CJD: an alternative cause related to post-industrial environmental contamination. *Med Hypotheses.* 2001. **57**, 555-560.

Brown, D.R., Hafiz, F., Glasssmith, L.L., Wong, B.S., Jones, I.M., Clive, C., Haswell, S.J. Consequences of manganese replacement of copper for prion protein function and proteinase resistance. *EMBO J.* 2000. **19**, 1180-1186.

Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., Kretzschmar, H. The cellular prion protein binds copper in vivo. *Nature.* 1997a. **390**, 684-687.

Brown, D.R., Schulz-Schaeffer, W.J., Schmidt, B., Kretzschmar, H.A. Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity. *Exp Neurol.* 1997b. **146**, 104-112.

Brown, D.R., Wong, B.S., Hafiz, F., Clive, C., Haswell, S.J., Jones, I.M. Normal prion protein has an activity like that of superoxide dismutase. *Biochem J.* 1999. **344 Pt 1**, 1-5.

Brown, P., Gajdusek, D.C. Survival of scrapie virus after 3 years' interment. *Lancet.* 1991. **337**, 269-270.

Bruce, M., Chree, A., McConnell, I., Foster, J., Pearson, G., Fraser, H. Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos Trans R Soc Lond B Biol Sci.* 1994. **343**, 405-411.

Bruce, M.E., Boyle, A., Cousens, S., McConnell, I., Foster, J., Goldmann, W., Fraser, H. Strain characterization of natural sheep scrapie and comparison with BSE. *J Gen Virol.* 2002. **83**, 695-704.

Bruce, M.E., McBride, P.A., Farquhar, C.F. Precise targeting of the pathology of the sialoglycoprotein, PrP, and vacuolar degeneration in mouse scrapie. *Neurosci Lett.* 1989. **102**, 1-6.

Bruce, M.E., Will, R.G., Ironside, J.W., McConnell, I., Drummond, D., Suttie, A., McCardle, L., Chree, A., Hope, J., Birkett, C., Cousens, S., Fraser, H., Bostock, C.J. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature.* 1997. **389**, 498-501.

Budka, H. Neuropathology of prion diseases. *Br Med Bull.* 2003. **66**, 121-130.

Buschmann, A., Kuczius, T., Bodemer, W., Groschup, M.H. Cellular prion proteins of mammalian species display an intrinsic partial proteinase K resistance. *Biochem Biophys Res Commun.* 1998. **253**, 693-702.

Cancellotti, E., Bradford, B.M., Tuzi, N.L., Hickey, R.D., Brown, D., Brown, K.L., Barron, R.M., Kisielewski, D., Piccardo, P., Manson, J.C. Glycosylation of PrPC determines timing of neuroinvasion and targeting in the brain following transmissible spongiform encephalopathy infection by a peripheral route. *J Virol.* 2010. **84**, 3464-3475.

Capobianco, R., Casalone, C., Suardi, S., Mangieri, M., Miccolo, C., Limido, L., Catania, M., Rossi, G., Di Fede, G., Giaccone, G., Bruzzone, M.G., Minati, L., Corona, C., Acutis, P., Gelmetti, D., Lombardi, G., Groschup, M.H., Buschmann, A., Zanusso, G., Monaco, S., Caramelli, M., Tagliavini, F. Conversion of the BASE prion strain into the BSE strain: the origin of BSE? *PLoS Pathog.* 2007. **3**, e31.

Carlson, G.A., Kingsbury, D.T., Goodman, P.A., Coleman, S., Marshall, S.T., DeArmond, S., Westaway, D., Prusiner, S.B. Linkage of prion protein and scrapie incubation time genes. *Cell.* 1986. **46**, 503-511.

Casalone, C., Zanusso, G., Acutis, P., Ferrari, S., Capucci, L., Tagliavini, F., Monaco, S., Caramelli, M. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. *Proc Natl Acad Sci U S A.* 2004. **101**, 3065-3070.

Caughey, B., Raymond, G.J., Kocisko, D.A., Lansbury, P.T., Jr. Scrapie infectivity correlates with converting activity, protease resistance, and aggregation of scrapie-associated prion protein in guanidine denaturation studies. *J Virol.* 1997. **71**, 4107-4110.

Chatelain, J., Cathala, F., Brown, P., Raharison, S., Court, L., Gajdusek, D.C. Epidemiologic comparisons between Creutzfeldt-Jakob disease and scrapie in France during the 12-year period 1968-1979. *J Neurol Sci.* 1981. **51**, 329-337.

Chen, S.G., Teplow, D.B., Parchi, P., Teller, J.K., Gambetti, P., Autilio-Gambetti, L. Truncated forms of the human prion protein in normal brain and in prion diseases. *J Biol Chem.* 1995. **270**, 19173-19180.

Cohen, F.E., Pan, K.M., Huang, Z., Baldwin, M., Fletterick, R.J., Prusiner, S.B. Structural clues to prion replication. *Science.* 1994. **264**, 530-531.

Collinge, J., Sidle, K.C., Meads, J., Ironside, J., Hill, A.F. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature.* 1996. **383**, 685-690.

Comoy, E.E., Casalone, C., Lescoutra-Etchegaray, N., Zanusso, G., Freire, S., Marce, D., Auvre, F., Ruchoux, M.M., Ferrari, S., Monaco, S., Sales, N., Caramelli, M., Leboulch, P., Brown, P., Lasmezas, C.I., Deslys, J.P. Atypical BSE (BASE) transmitted from asymptomatic aging cattle to a primate. *PLoS One.* 2008. **3**, e3017.

Cooke, C.M., Rodger, J., Smith, A., Fernie, K., Shaw, G., Somerville, R.A. Fate of prions in soil: detergent extraction of PrP from soils. *Environ Sci Technol.* 2007. **41**, 811-817.

Cordeiro, Y., Kraineva, J., Gomes, M.P., Lopes, M.H., Martins, V.R., Lima, L.M., Foguel, D., Winter, R., Silva, J.L. The amino-terminal PrP domain is crucial to modulate prion misfolding and aggregation. *Biophys J.* 2005. **89**, 2667-2676.

Cui, T., Daniels, M., Wong, B.S., Li, R., Sy, M.S., Sassoon, J., Brown, D.R. Mapping the functional domain of the prion protein. *Eur J Biochem.* 2003. **270**, 3368-3376.

David Walter, W., Walsh, D.P., Farnsworth, M.L., Winkelman, D.L., Miller, M.W. Soil clay content underlies prion infection odds. *Nat Commun.* 2011. **2**, 200.

Davies, P., Brown, D.R. Manganese enhances prion protein survival in model soils and increases prion infectivity to cells. *PLoS One.* 2009. **4**, e7518.

DeArmond, S.J., Mobley, W.C., DeMott, D.L., Barry, R.A., Beckstead, J.H., Prusiner, S.B. Changes in the localization of brain prion proteins during scrapie infection. *Neurology.* 1987. **37**, 1271-1280.

DeArmond, S.J., Yang, S.L., Lee, A., Bowler, R., Taraboulos, A., Groth, D., Prusiner, S.B. Three scrapie prion isolates exhibit different accumulation patterns of the prion protein scrapie isoform. *Proc Natl Acad Sci U S A.* 1993. **90**, 6449-6453.

Deleault, N.R., Kascsak, R., Geoghegan, J.C., Supattapone, S. Species-dependent differences in cofactor utilization for formation of the protease-resistant prion protein in vitro. *Biochemistry.* 2010. **49**, 3928-3934.

Deleault, N.R., Lucassen, R.W., Supattapone, S. RNA molecules stimulate prion protein conversion. *Nature.* 2003. **425**, 717-720.

Denkers, N.D., Telling, G.C., Hoover, E.A. Minor oral lesions facilitate transmission of chronic wasting disease. *J Virol.* 2011. **85**, 1396-1399.

Dickinson, A.G., Outram, G.W. Genetic aspects of unconventional virus infections: the basis of the virino hypothesis. *Ciba Found Symp.* 1988. **135**, 63-83.

Doherr, M.G. Bovine spongiform encephalopathy (BSE)--infectious, contagious, zoonotic or production disease? *Acta Vet Scand Suppl.* 2003. **98**, 33-42.

Dron, M., Moudjou, M., Chapuis, J., Salamat, M.K., Bernard, J., Cronier, S., Langevin, C., Laude, H. Endogenous proteolytic cleavage of disease-associated prion protein to produce C2 fragments is strongly cell- and tissue-dependent. *J Biol Chem.* 2010. **285**, 10252-10264.

Elsen, J.M., Amigues, Y., Schelcher, F., Ducrocq, V., Andreoletti, O., Eychenne, F., Khang, J.V., Poivey, J.P., Lantier, F., Laplanche, J.L. Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. *Arch Virol.* 1999. **144**, 431-445.

Endo, T., Groth, D., Prusiner, S.B., Kobata, A. Diversity of oligosaccharide structures linked to asparagines of the scrapie prion protein. *Biochemistry.* 1989. **28**, 8380-8388.

Espinosa, J.C., Herva, M.E., Andreoletti, O., Padilla, D., Lacroux, C., Cassard, H., Lantier, I., Castilla, J., Torres, J.M. Transgenic mice expressing porcine prion protein resistant to classical scrapie but susceptible to sheep bovine spongiform encephalopathy and atypical scrapie. *Emerg Infect Dis.* 2009. **15**, 1214-1221.

Espinosa, J.C., Morales, M., Castilla, J., Rogers, M., Torres, J.M. Progression of prion infectivity in asymptomatic cattle after oral bovine spongiform encephalopathy challenge. *J Gen Virol.* 2007. **88**, 1379-1383.

Flechsig, E., Shmerling, D., Hegyi, I., Raeber, A.J., Fischer, M., Cozzio, A., von Mering, C., Aguzzi, A., Weissmann, C. Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron.* 2000. **27**, 399-408.

Fraser, H., Dickinson, A.G. The sequential development of the brain lesion of scrapie in three strains of mice. *J Comp Pathol.* 1968. **78**, 301-311.

Fraser, H., Dickinson, A.G. Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation. *J Comp Pathol.* 1973. **83**, 29-40.

Gabriel, J.M., Oesch, B., Kretzschmar, H., Scott, M., Prusiner, S.B. Molecular cloning of a candidate chicken prion protein. *Proc Natl Acad Sci U S A.* 1992. **89**, 9097-9101.

Gambetti, P., Dong, Z., Yuan, J., Xiao, X., Zheng, M., Alshekhlee, A., Castellani, R., Cohen, M., Barria, M.A., Gonzalez-Romero, D., Belay, E.D., Schonberger, L.B., Marder, K., Harris, C., Burke, J.R., Montine, T., Wisniewski, T., Dickson, D.W., Soto, C., Hulette, C.M., Mastrianni, J.A., Kong, Q., Zou, W.Q. A novel human disease with abnormal prion protein sensitive to protease. *Ann Neurol.* 2008. **63**, 697-708.

Georgsson, G., Sigurdarson, S., Brown, P. Infectious agent of sheep scrapie may persist in the environment for at least 16 years. *J Gen Virol.* 2006. **87**, 3737-3740.

Groschup, M.H., Lacroux, C., Buschmann, A., Luhken, G., Mathey, J., Eiden, M., Lugan, S., Hoffmann, C., Espinosa, J.C., Baron, T., Torres, J.M., Erhardt, G., Andreoletti, O. Classic scrapie in sheep with the ARR/ARR prion genotype in Germany and France. *Emerg Infect Dis.* 2007. **13**, 1201-1207.

Hainfellner, J.A., Brantner-Inthaler, S., Cervenakova, L., Brown, P., Kitamoto, T., Tateishi, J., Diringer, H., Liberski, P.P., Regele, H., Feucht, M., et al. The original Gerstmann-Straussler-Scheinker family of Austria: divergent clinicopathological phenotypes but constant PrP genotype. *Brain Pathol.* 1995. **5**, 201-211.

Haley, N.J., Mathiason, C.K., Zabel, M.D., Telling, G.C., Hoover, E.A. Detection of sub-clinical CWD infection in conventional test-negative deer long after oral exposure to urine and feces from CWD+ deer. *PLoS One.* 2009a. **4**, e7990.

Haley, N.J., Seelig, D.M., Zabel, M.D., Telling, G.C., Hoover, E.A. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. *PLoS One.* 2009b. **4**, e4848.

Hamir, A.N., Cutlip, R.C., Miller, J.M., Williams, E.S., Stack, M.J., Miller, M.W., O'Rourke, K.I., Chaplin, M.J. Preliminary findings on the experimental transmission of chronic wasting disease agent of mule deer to cattle. *J Vet Diagn Invest.* 2001. **13**, 91-96.

Hamir, A.N., Kunkle, R.A., Cutlip, R.C., Miller, J.M., Williams, E.S., Richt, J.A. Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation. *J Vet Diagn Invest.* 2006. **18**, 558-565.

Hamir, A.N., Kunkle, R.A., Richt, J.A., Miller, J.M., Greenlee, J.J. Experimental transmission of US scrapie agent by nasal, peritoneal, and conjunctival routes to genetically susceptible sheep. *Vet Pathol.* 2008. **45**, 7-11.

Heppner, F.L., Christ, A.D., Klein, M.A., Prinz, M., Fried, M., Kraehenbuhl, J.P., Aguzzi, A. Transepithelial prion transport by M cells. *Nat Med.* 2001. **7**, 976-977.

Hill, A.F., Desbruslais, M., Joiner, S., Sidle, K.C., Gowland, I., Collinge, J., Doey, L.J., Lantos, P. The same prion strain causes vCJD and BSE. *Nature.* 1997. **389**, 448-450, 526.

Hill, A.F., Joiner, S., Wadsworth, J.D., Sidle, K.C., Bell, J.E., Budka, H., Ironside, J.W., Collinge, J. Molecular classification of sporadic Creutzfeldt-Jakob disease. *Brain.* 2003. **126**, 1333-1346.

Hill, A.F., Sidle, K.C., Joiner, S., Keyes, P., Martin, T.C., Dawson, M., Collinge, J. Molecular screening of sheep for bovine spongiform encephalopathy. *Neurosci Lett.* 1998. **255**, 159-162.

Hilton, D.A., Ghani, A.C., Conyers, L., Edwards, P., McCardle, L., Penney, M., Ritchie, D., Ironside, J.W. Accumulation of prion protein in tonsil and appendix: review of tissue samples. *BMJ.* 2002. **325**, 633-634.

Hoinville, L.J. A review of the epidemiology of scrapie in sheep. *Rev Sci Tech.* 1996. **15**, 827-852.

Hope, J., Morton, L.J., Farquhar, C.F., Multhaup, G., Beyreuther, K., Kimberlin, R.H. The major polypeptide of scrapie-associated fibrils (SAF) has the same size, charge distribution and N-terminal protein sequence as predicted for the normal brain protein (PrP). *EMBO J.* 1986. **5**, 2591-2597.

Hope, J., Multhaup, G., Reekie, L.J., Kimberlin, R.H., Beyreuther, K. Molecular pathology of scrapie-associated fibril protein (PrP) in mouse brain affected by the ME7 strain of scrapie. *Eur J Biochem.* 1988. **172**, 271-277.

Hope, J., Wood, S.C., Birkett, C.R., Chong, A., Bruce, M.E., Cairns, D., Goldmann, W., Hunter, N., Bostock, C.J. Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. *J Gen Virol.* 1999. **80 (Pt 1)**, 1-4.

Houston, F., McCutcheon, S., Goldmann, W., Chong, A., Foster, J., Siso, S., Gonzalez, L., Jeffrey, M., Hunter, N. Prion diseases are efficiently transmitted by blood transfusion in sheep. *Blood.* 2008. **112**, 4739-4745.

Hui, C.A. Geophagy and potential contaminant exposure for terrestrial vertebrates. *Rev Environ Contam Toxicol.* 2004. **183**, 115-134.

Hunter, N., Foster, J., Chong, A., McCutcheon, S., Parnham, D., Eaton, S., MacKenzie, C., Houston, F. Transmission of prion diseases by blood transfusion. *J Gen Virol.* 2002. **83**, 2897-2905.

Hunter, N., Goldmann, W., Smith, G., Hope, J. Frequencies of PrP gene variants in healthy cattle and cattle with BSE in Scotland. *Vet Rec.* 1994. **135**, 400-403.

Jacobson, K.H., Lee, S., Somerville, R.A., McKenzie, D., Benson, C.H., Pedersen, J.A. Transport of the pathogenic prion protein through soils. *J Environ Qual.* 2010. **39**, 1145-1152.

Jeffrey, M., Begara-McGorum, I., Clark, S., Martin, S., Clark, J., Chaplin, M., Gonzalez, L. Occurrence and distribution of infection-specific PrP in tissues of clinical scrapie cases and cull sheep from scrapie-affected farms in Shetland. *J Comp Pathol.* 2002. **127**, 264-273.

Jeffrey, M., Gonzalez, L. Classical sheep transmissible spongiform encephalopathies: pathogenesis, pathological phenotypes and clinical disease. *Neuropathol Appl Neurobiol.* 2007. **33**, 373-394.

Jeffrey, M., Gonzalez, L., Chong, A., Foster, J., Goldmann, W., Hunter, N., Martin, S. Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunochemical similarities can be resolved by immunohistochemistry. *J Comp Pathol.* 2006a. **134**, 17-29.

Jeffrey, M., Martin, S., Gonzalez, L., Foster, J., Langeveld, J.P., van Zijderveld, F.G., Grassi, J., Hunter, N. Immunohistochemical features of PrP(d) accumulation in natural and experimental goat transmissible spongiform encephalopathies. *J Comp Pathol.* 2006b. **134**, 171-181.

Jeffrey, M., Martin, S., Gonzalez, L., Ryder, S.J., Bellworthy, S.J., Jackman, R. Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. *J Comp Pathol.* 2001. **125**, 271-284.

Johnson, C.J., Pedersen, J.A., Chappell, R.J., McKenzie, D., Aiken, J.M. Oral transmissibility of prion disease is enhanced by binding to soil particles. *PLoS Pathog.* 2007. **3**, e93.

Johnson, C.J., Phillips, K.E., Schramm, P.T., McKenzie, D., Aiken, J.M., Pedersen, J.A. Prions adhere to soil minerals and remain infectious. *PLoS Pathog.* 2006. **2**, e32.

Jones, C.E., Klewpatinond, M., Abdelraheim, S.R., Brown, D.R., Viles, J.H. Probing copper2+ binding to the prion protein using diamagnetic nickel2+ and 1H NMR: the unstructured N terminus facilitates the coordination of six copper2+ ions at physiological concentrations. *J Mol Biol.* 2005a. **346**, 1393-1407.

Jones, S., Batchelor, M., Bhelt, D., Clarke, A.R., Collinge, J., Jackson, G.S. Recombinant prion protein does not possess SOD-1 activity. *Biochem J.* 2005b. **392**, 309-312.

Kaski, D., Mead, S., Hyare, H., Cooper, S., Jampana, R., Overell, J., Knight, R., Collinge, J., Rudge, P. Variant CJD in an individual heterozygous for PRNP codon 129. *Lancet.* 2009. **374**, 2128.

Konold, T., Spiropoulos, J., Chaplin, M.J., Thorne, L., Spencer, Y.I., Wells, G.A., Hawkins, S.A. Transmissibility studies of vacuolar changes in the rostral colliculus of pigs. *BMC Vet Res.* 2009. **5**, 35.

Korth, C., Kaneko, K., Prusiner, S.B. Expression of unglycosylated mutated prion protein facilitates PrP(Sc) formation in neuroblastoma cells infected with different prion strains. *J Gen Virol.* 2000. **81**, 2555-2563.

Kuczius, T., Buschmann, A., Zhang, W., Karch, H., Becker, K., Peters, G., Groschup, M.H. Cellular prion protein acquires resistance to proteolytic degradation following copper ion binding. *Biol Chem.* 2004. **385**, 739-747.

Kupfer, L., Eiden, M., Buschmann, A., Groschup, M.H. Amino acid sequence and prion strain specific effects on the in vitro and in vivo convertibility of ovine/murine and bovine/murine prion protein chimeras. *Biochim Biophys Acta.* 2007. **1772**, 704-713.

Lasmezas, C.I., Deslys, J.P., Demaimay, R., Adjou, K.T., Lamoury, F., Dormont, D., Robain, O., Ironside, J., Hauw, J.J. BSE transmission to macaques. *Nature.* 1996. **381**, 743-744.

Lasmezas, C.I., Deslys, J.P., Robain, O., Jaegly, A., Beringue, V., Peyrin, J.M., Fournier, J.G., Hauw, J.J., Rossier, J., Dormont, D. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science.* 1997. **275**, 402-405.

Laws, D.D., Bitter, H.M., Liu, K., Ball, H.L., Kaneko, K., Wille, H., Cohen, F.E., Prusiner, S.B., Pines, A., Wemmer, D.E. Solid-state NMR studies of the secondary structure of a mutant prion protein fragment of 55 residues that induces neurodegeneration. *Proc Natl Acad Sci U S A.* 2001. **98**, 11686-11690.

Lawson, V.A., Furness, J.B., Klemm, H.M., Pontell, L., Chan, E., Hill, A.F., Chiocchetti, R. The brain to gut pathway: a possible route of prion transmission. *Gut.* 2010. **59**, 1643-1651.

Le Dur, A., Beringue, V., Andreoletti, O., Reine, F., Lai, T.L., Baron, T., Bratberg, B., Vilotte, J.L., Sarradin, P., Benestad, S.L., Laude, H. A newly identified type of scrapie agent can naturally infect sheep with resistant PrP genotypes. *Proc Natl Acad Sci U S A.* 2005. **102**, 16031-16036.

Lezmi, S., Martin, S., Simon, S., Comoy, E., Bencsik, A., Deslys, J.P., Grassi, J., Jeffrey, M., Baron, T. Comparative molecular analysis of the abnormal prion protein in field scrapie cases and experimental bovine spongiform encephalopathy in sheep by use of Western blotting and immunohistochemical methods. *J Virol.* 2004. **78**, 3654-3662.

Lindenbaum, S. Review. Understanding kuru: the contribution of anthropology and medicine. *Philos Trans R Soc Lond B Biol Sci.* 2008. **363**, 3715-3720.

Locht, C., Chesebro, B., Race, R., Keith, J.M. Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent. *Proc Natl Acad Sci U S A.* 1986. **83**, 6372-6376.

Ma, X., Benson, C.H., McKenzie, D., Aiken, J.M., Pedersen, J.A. Adsorption of pathogenic prion protein to quartz sand. *Environ Sci Technol.* 2007. **41**, 2324-2330.

Maddison, B.C., Baker, C.A., Rees, H.C., Terry, L.A., Thorne, L., Bellworthy, S.J., Whitelam, G.C., Gough, K.C. Prions are secreted in milk from clinically normal scrapie-exposed sheep. *J Virol.* 2009. **83**, 8293-8296.

Maddison, B.C., Baker, C.A., Terry, L.A., Bellworthy, S.J., Thorne, L., Rees, H.C., Gough, K.C. Environmental sources of scrapie prions. *J Virol.* 2010a. **84**, 11560-11562.

Maddison, B.C., Owen, J.P., Bishop, K., Shaw, G., Rees, H.C., Gough, K.C. The interaction of ruminant PrP(Sc) with soils is influenced by prion source and soil type. *Environ Sci Technol.* 2010b. **44**, 8503-8508.

Maddison, B.C., Rees, H.C., Baker, C.A., Taema, M., Bellworthy, S.J., Thorne, L., Terry, L.A., Gough, K.C. Prions are secreted into the oral cavity in sheep with preclinical scrapie. *J Infect Dis.* 2010c. **201**, 1672-1676.

Mahal, S.P., Baker, C.A., Demczyk, C.A., Smith, E.W., Julius, C., Weissmann, C. Prion strain discrimination in cell culture: the cell panel assay. *Proc Natl Acad Sci U S A.* 2007. **104**, 20908-20913.

Mahal, S.P., Browning, S., Li, J., Suponitsky-Kroyter, I., Weissmann, C. Transfer of a prion strain to different hosts leads to emergence of strain variants. *Proc Natl Acad Sci U S A.* 2010. **107**, 22653-22658.

Manuelidis, L. A 25 nm virion is the likely cause of transmissible spongiform encephalopathies. *J Cell Biochem.* 2007. **100**, 897-915.

Manuelidis, L., Liu, Y., Mullins, B. Strain-specific viral properties of variant Creutzfeldt-Jakob disease (vCJD) are encoded by the agent and not by host prion protein. *J Cell Biochem.* 2009. **106**, 220-231.

Manuelidis, L., Yu, Z.X., Barquero, N., Mullins, B. Cells infected with scrapie and Creutzfeldt-Jakob disease agents produce intracellular 25-nm virus-like particles. *Proc Natl Acad Sci U S A.* 2007. **104**, 1965-1970.

Masel, J., Jansen, V.A., Nowak, M.A. Quantifying the kinetic parameters of prion replication. *Biophys Chem.* 1999. **77**, 139-152.

Mathiason, C.K., Hayes-Klug, J., Hays, S.A., Powers, J., Osborn, D.A., Dahmes, S.J., Miller, K.V., Warren, R.J., Mason, G.L., Telling, G.C., Young, A.J., Hoover, E.A. B cells

and platelets harbor prion infectivity in the blood of deer infected with chronic wasting disease. *J Virol.* 2010. **84**, 5097-5107.

Mathiason, C.K., Hays, S.A., Powers, J., Hayes-Klug, J., Langenberg, J., Dahmes, S.J., Osborn, D.A., Miller, K.V., Warren, R.J., Mason, G.L., Hoover, E.A. Infectious prions in pre-clinical deer and transmission of chronic wasting disease solely by environmental exposure. *PLoS One.* 2009. **4**, e5916.

Mathiason, C.K., Powers, J.G., Dahmes, S.J., Osborn, D.A., Miller, K.V., Warren, R.J., Mason, G.L., Hays, S.A., Hayes-Klug, J., Seelig, D.M., Wild, M.A., Wolfe, L.L., Spraker, T.R., Miller, M.W., Sigurdson, C.J., Telling, G.C., Hoover, E.A. Infectious prions in the saliva and blood of deer with chronic wasting disease. *Science.* 2006. **314**, 133-136.

Mawhinney, S., Pape, W.J., Forster, J.E., Anderson, C.A., Bosque, P., Miller, M.W. Human prion disease and relative risk associated with chronic wasting disease. *Emerg Infect Dis.* 2006. **12**, 1527-1535.

Mayland, H.F., Shewmaker, G.E., Bull, R.C. Soil Ingestion by Cattle Grazing Crested Wheatgrass. *Journal of Range Management.* 1977. **30**, 2.

Mazza, M., Iulini, B., Vaccari, G., Acutis, P.L., Martucci, F., Esposito, E., Peletto, S., Barocci, S., Chiappini, B., Corona, C., Barbieri, I., Caramelli, M., Agrimi, U., Casalone, C., Nonno, R. Co-existence of classical scrapie and Nor98 in a sheep from an Italian outbreak. *Res Vet Sci.* 2010. **88**, 478-485.

McGowan, J.P. Scrapie in sheep. *Scottish J Agric.* 1922. **5**, 365-375.

McKinley, M.P., Bolton, D.C., Prusiner, S.B. A protease-resistant protein is a structural component of the scrapie prion. *Cell.* 1983. **35**, 57-62.

Miller, J.M., Jenny, A.L., Taylor, W.D., Marsh, R.F., Rubenstein, R., Race, R.E. Immunohistochemical detection of prion protein in sheep with scrapie. *J Vet Diagn Invest.* 1993. **5**, 309-316.

Miller, M.W., Williams, E.S., Hobbs, N.T., Wolfe, L.L. Environmental sources of prion transmission in mule deer. *Emerg Infect Dis.* 2004. **10**, 1003-1006.

Mishra, R.S., Basu, S., Gu, Y., Luo, X., Zou, W.Q., Mishra, R., Li, R., Chen, S.G., Gambetti, P., Fujioka, H., Singh, N. Protease-resistant human prion protein and ferritin are

cotransported across Caco-2 epithelial cells: implications for species barrier in prion uptake from the intestine. *J Neurosci.* 2004. **24**, 11280-11290.

Mitteregger, G., Korte, S., Shakarami, M., Herms, J., Kretzschmar, H.A. Role of copper and manganese in prion disease progression. *Brain Res.* 2009. **1292**, 155-164.

Mohan, J., Brown, K.L., Farquhar, C.F., Bruce, M.E., Mabbott, N.A. Scrapie transmission following exposure through the skin is dependent on follicular dendritic cells in lymphoid tissues. *J Dermatol Sci.* 2004. **35**, 101-111.

Moore, R.A., Herzog, C., Errett, J., Kocisko, D.A., Arnold, K.M., Hayes, S.F., Priola, S.A. Octapeptide repeat insertions increase the rate of protease-resistant prion protein formation. *Protein Sci.* 2006. **15**, 609-619.

Murayama, Y., Yoshioka, M., Okada, H., Takata, M., Yokoyama, T., Mohri, S. Urinary excretion and blood level of prions in scrapie-infected hamsters. *J Gen Virol.* 2007. **88**, 2890-2898.

Nathanson, N., Wilesmith, J., Griot, C. Bovine spongiform encephalopathy (BSE): causes and consequences of a common source epidemic. *Am J Epidemiol.* 1997. **145**, 959-969.

Nicot, S., Baron, T.G. Strain-specific proteolytic processing of the prion protein in prion diseases of ruminants transmitted in ovine transgenic mice. *J Gen Virol.* 2010. **91**, 570-574.

Notari, S., Capellari, S., Giese, A., Westner, I., Baruzzi, A., Ghetti, B., Gambetti, P., Kretzschmar, H.A., Parchi, P. Effects of different experimental conditions on the PrPSc core generated by protease digestion: implications for strain typing and molecular classification of CJD. *J Biol Chem.* 2004. **279**, 16797-16804.

Nunziante, M., Gilch, S., Schatzl, H.M. Essential role of the prion protein N terminus in subcellular trafficking and half-life of cellular prion protein. *J Biol Chem.* 2003. **278**, 3726-3734.

Owen, J.P., Maddison, B.C., Whitelam, G.C., Gough, K.C. Use of thermolysin in the diagnosis of prion diseases. *Mol Biotechnol.* 2007. **35**, 161-170.

Padilla, D., Beringue, V., Espinosa, J.C., Andreoletti, O., Jaumain, E., Reine, F., Herzog, L., Gutierrez-Adan, A., Pintado, B., Laude, H., Torres, J.M. Sheep and goat BSE

propagate more efficiently than cattle BSE in human PrP transgenic mice. *PLoS Pathog.* 2011. **7**, e1001319.

Pan, K.M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R.J., Cohen, F.E., et al. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A.* 1993. **90**, 10962-10966.

Pan, T., Wong, P., Chang, B., Li, C., Li, R., Kang, S.C., Wisniewski, T., Sy, M.S. Biochemical fingerprints of prion infection: accumulations of aberrant full-length and N-terminally truncated PrP species are common features in mouse prion disease. *J Virol.* 2005. **79**, 934-943.

Pattison, I.H., Jones, K.M. The possible nature of the transmissible agent of scrapie. *Vet Rec.* 1967. **80**, 2-9.

Pattison, I.H., Millson, G.C. Scrapie produced experimentally in goats with special reference to the clinical syndrome. *J Comp Pathol.* 1961. **71**, 101-109.

Peden, A.H., Head, M.W., Ritchie, D.L., Bell, J.E., Ironside, J.W. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet.* 2004. **364**, 527-529.

Peretz, D., Williamson, R.A., Legname, G., Matsunaga, Y., Vergara, J., Burton, D.R., DeArmond, S.J., Prusiner, S.B., Scott, M.R. A change in the conformation of prions accompanies the emergence of a new prion strain. *Neuron.* 2002. **34**, 921-932.

Pirisinu, L., Migliore, S., Di Bari, M.A., Esposito, E., Baron, T., D'Agostino, C., De Grossi, L., Vaccari, G., Agrimi, U., Nonno, R. Molecular discrimination of sheep bovine spongiform encephalopathy from scrapie. *Emerg Infect Dis.* 2011. **17**, 695-698.

Piro, J.R., Harris, B.T., Nishina, K., Soto, C., Morales, R., Rees, J.R., Supattapone, S. Prion protein glycosylation is not required for strain-specific neurotropism. *J Virol.* 2009. **83**, 5321-5328.

Prusiner, S.B. Novel proteinaceous infectious particles cause scrapie. *Science.* 1982. **216**, 136-144.

Prusiner, S.B. Molecular biology of prion diseases. *Science.* 1991. **252**, 1515-1522.

Prusiner, S.B. Prions. *Proc Natl Acad Sci U S A.* 1998. **95**, 13363-13383.

Purdey, M. High-dose exposure to systemic phosmet insecticide modifies the phosphatidylinositol anchor on the prion protein: the origins of new variant transmissible spongiform encephalopathies? *Med Hypotheses.* 1998. **50**, 91-111.

Race, B., Meade-White, K.D., Miller, M.W., Barbian, K.D., Rubenstein, R., LaFauci, G., Cervenakova, L., Favara, C., Gardner, D., Long, D., Parnell, M., Striebel, J., Priola, S.A., Ward, A., Williams, E.S., Race, R., Chesebro, B. Susceptibilities of nonhuman primates to chronic wasting disease. *Emerg Infect Dis.* 2009. **15**, 1366-1376.

Rescigno, M., Rotta, G., Valzasina, B., Ricciardi-Castagnoli, P. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology.* 2001. **204**, 572-581.

Riek, R., Hornemann, S., Wider, G., Billeter, M., Glockshuber, R., Wuthrich, K. NMR structure of the mouse prion protein domain PrP(121-321). *Nature.* 1996. **382**, 180-182.

Russo, F., Johnson, C.J., McKenzie, D., Aiken, J.M., Pedersen, J.A. Pathogenic prion protein is degraded by a manganese oxide mineral found in soils. *J Gen Virol.* 2009. **90**, 275-280.

Saborio, G.P., Permanne, B., Soto, C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature.* 2001. **411**, 810-813.

Safar, J., Wang, W., Padgett, M.P., Ceroni, M., Piccardo, P., Zopf, D., Gajdusek, D.C., Gibbs, C.J., Jr. Molecular mass, biochemical composition, and physicochemical behavior of the infectious form of the scrapie precursor protein monomer. *Proc Natl Acad Sci U S A.* 1990. **87**, 6373-6377.

Safar, J.G., Lessard, P., Tamguney, G., Freyman, Y., Deering, C., Letessier, F., Dearmond, S.J., Prusiner, S.B. Transmission and detection of prions in feces. *J Infect Dis.* 2008. **198**, 81-89.

Sandberg, M.K., Al-Doujaily, H., Sigurdson, C.J., Glatzel, M., O'Malley, C., Powell, C., Asante, E.A., Linehan, J.M., Brandner, S., Wadsworth, J.D., Collinge, J. Chronic wasting disease prions are not transmissible to transgenic mice overexpressing human prion protein. *J Gen Virol.* 2010. **91**, 2651-2657.

Saunders, S.E., Bartz, J.C., Bartelt-Hunt, S.L. Influence of prion strain on prion protein adsorption to soil in a competitive matrix. *Environ Sci Technol.* 2009a. **43**, 5242-5248.

Saunders, S.E., Bartz, J.C., Bartelt-Hunt, S.L. Prion protein adsorption to soil in a competitive matrix is slow and reduced. *Environ Sci Technol.* 2009b. **43**, 7728-7733.

Saunders, S.E., Bartz, J.C., Telling, G.C., Bartelt-Hunt, S.L. Environmentally-relevant forms of the prion protein. *Environ Sci Technol.* 2008. **42**, 6573-6579.

Saunders, S.E., Bartz, J.C., Vercauteren, K.C., Bartelt-Hunt, S.L. Enzymatic digestion of chronic wasting disease prions bound to soil. *Environ Sci Technol.* 2010. **44**, 4129-4135.

Schreuder, B.E., van Keulen, L.J., Vromans, M.E., Langeveld, J.P., Smits, M.A. Preclinical test for prion diseases. *Nature.* 1996. **381**, 563.

Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Walchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S.J., Westaway, D., Prusiner, S.B. Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell.* 1989. **59**, 847-857.

Seidel, B., Thomzig, A., Buschmann, A., Groschup, M.H., Peters, R., Beekes, M., Terytze, K. Scrapie Agent (Strain 263K) can transmit disease via the oral route after persistence in soil over years. *PLoS One.* 2007. **2**, e435.

Sekijima, M., Motono, C., Yamasaki, S., Kaneko, K., Akiyama, Y. Molecular dynamics simulation of dimeric and monomeric forms of human prion protein: insight into dynamics and properties. *Biophys J.* 2003. **85**, 1176-1185.

Sigurdson, C.J., Miller, M.W. Other animal prion diseases. *Br Med Bull.* 2003. **66**, 199-212.

Simmons, H.A., Simmons, M.M., Spencer, Y.I., Chaplin, M.J., Povey, G., Davis, A., Ortiz-Pelaez, A., Hunter, N., Matthews, D., Wrathall, A.E. Atypical scrapie in sheep from a UK research flock which is free from classical scrapie. *BMC Vet Res.* 2009. **5**, 8.

Smith, P.G., Bradley, R. Bovine spongiform encephalopathy (BSE) and its epidemiology. *Br Med Bull.* 2003. **66**, 185-198.

Spiro, R.G. Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology.* 2002. **12**, 43R-56R.

Stack, M., Jeffrey, M., Gubbins, S., Grimmer, S., Gonzalez, L., Martin, S., Chaplin, M., Webb, P., Simmons, M., Spencer, Y., Bellerby, P., Hope, J., Wilesmith, J., Matthews, D. Monitoring for bovine spongiform encephalopathy in sheep in Great Britain, 1998-2004. *J Gen Virol.* 2006. **87**, 2099-2107.

Stack, M.J., Chaplin, M.J., Clark, J. Differentiation of prion protein glycoforms from naturally occurring sheep scrapie, sheep-passaged scrapie strains (CH1641 and SSBP1), bovine spongiform encephalopathy (BSE) cases and Romney and Cheviot breed sheep experimentally inoculated with BSE using two monoclonal antibodies. *Acta Neuropathol.* 2002. **104**, 279-286.

Stack, M.J., Focosi-Snyman, R., Cawthraw, S., Davis, L., Chaplin, M.J., Burke, P.J. Third atypical BSE case in Great Britain with an H-type molecular profile. *Vet Rec.* 2009. **165**, 605-606.

Stevens, D.J., Walter, E.D., Rodriguez, A., Draper, D., Davies, P., Brown, D.R., Millhauser, G.L. Early onset prion disease from octarepeat expansion correlates with copper binding properties. *PLoS Pathog.* 2009. **5**, e1000390.

Taema, M.M., Maddison, B.C., Thorne, L., Bishop, K., Owen, J., Hunter, N., Baker, C.A., Terry, L.A., Gough, K.C. Differentiating Ovine BSE from CH1641 Scrapie by Serial Protein Misfolding Cyclic Amplification. *Mol Biotechnol.* 2011.

Tamguney, G., Miller, M.W., Wolfe, L.L., Sirochman, T.M., Glidden, D.V., Palmer, C., Lemus, A., DeArmond, S.J., Prusiner, S.B. Asymptomatic deer excrete infectious prions in faeces. *Nature.* 2009. **461**, 529-532.

Taraboulos, A., Rogers, M., Borchelt, D.R., McKinley, M.P., Scott, M., Serban, D., Prusiner, S.B. Acquisition of protease resistance by prion proteins in scrapie-infected cells does not require asparagine-linked glycosylation. *Proc Natl Acad Sci U S A.* 1990. **87**, 8262-8266.

Taylor, D.M., Fernie, K., McConnell, I., Ferguson, C.E., Steele, P.J. Solvent extraction as an adjunct to rendering: the effect on BSE and scrapie agents of hot solvents followed by dry heat and steam. *Vet Rec.* 1998. **143**, 6-9.

Telling, G.C., Scott, M., Hsiao, K.K., Foster, D., Yang, S.L., Torchia, M., Sidle, K.C., Collinge, J., DeArmond, S.J., Prusiner, S.B. Transmission of Creutzfeldt-Jakob disease

from humans to transgenic mice expressing chimeric human-mouse prion protein. *Proc Natl Acad Sci U S A.* 1994. **91**, 9936-9940.

Terry, L.A., Howells, L., Hawthorn, J., Edwards, J.C., Moore, S.J., Bellworthy, S.J., Simmons, H., Lizano, S., Estey, L., Leathers, V., Everest, S.J. Detection of PrPsc in blood from sheep infected with the scrapie and bovine spongiform encephalopathy agents. *J Virol.* 2009. **83**, 12552-12558.

Thackray, A.M., Hopkins, L., Lockey, R., Spiropoulos, J., Bujdoso, R. Emergence of multiple prion strains from single isolates of ovine scrapie. *J Gen Virol.* 2011. **92**, 1482-1491.

Thackray, A.M., Knight, R., Haswell, S.J., Bujdoso, R., Brown, D.R. Metal imbalance and compromised antioxidant function are early changes in prion disease. *Biochem J.* 2002. **362**, 253-258.

Tobler, I., Gaus, S.E., Deboer, T., Achermann, P., Fischer, M., Rulicke, T., Moser, M., Oesch, B., McBride, P.A., Manson, J.C. Altered circadian activity rhythms and sleep in mice devoid of prion protein. *Nature.* 1996. **380**, 639-642.

Tuzi, N.L., Cancellotti, E., Baybutt, H., Blackford, L., Bradford, B., Plinston, C., Coghill, A., Hart, P., Piccardo, P., Barron, R.M., Manson, J.C. Host PrP glycosylation: a major factor determining the outcome of prion infection. *PLoS Biol.* 2008. **6**, e100.

van Keulen, L.J., Bossers, A., van Zijderveld, F. TSE pathogenesis in cattle and sheep. *Vet Res.* 2008. **39**, 24.

Vilotte, J.L., Soulier, S., Essalmani, R., Stinnakre, M.G., Vaiman, D., Lepourry, L., Da Silva, J.C., Besnard, N., Dawson, M., Buschmann, A., Groschup, M., Petit, S., Madelaine, M.F., Rakatobe, S., Le Dur, A., Vilette, D., Laude, H. Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine prp. *J Virol.* 2001. **75**, 5977-5984.

Vorberg, I., Priola, S.A. Molecular basis of scrapie strain glycoform variation. *J Biol Chem.* 2002. **277**, 36775-36781.

Walmsley, A.R., Zeng, F., Hooper, N.M. Membrane topology influences N-glycosylation of the prion protein. *EMBO J.* 2001. **20**, 703-712.

Wang, F., Wang, X., Yuan, C.G., Ma, J. Generating a prion with bacterially expressed recombinant prion protein. *Science.* 2010. **327**, 1132-1135.

Wells, G.A., Hawkins, S.A., Austin, A.R., Ryder, S.J., Done, S.H., Green, R.B., Dexter, I., Dawson, M., Kimberlin, R.H. Studies of the transmissibility of the agent of bovine spongiform encephalopathy to pigs. *J Gen Virol.* 2003. **84**, 1021-1031.

Westergard, L., Christensen, H.M., Harris, D.A. The cellular prion protein (PrP(C)): its physiological function and role in disease. *Biochim Biophys Acta.* 2007. **1772**, 629-644.

Wilesmith, J.W., Ryan, J.B., Atkinson, M.J. Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet Rec.* 1991. **128**, 199-203.

Wille, H., Zhang, G.F., Baldwin, M.A., Cohen, F.E., Prusiner, S.B. Separation of scrapie prion infectivity from PrP amyloid polymers. *J Mol Biol.* 1996. **259**, 608-621.

Williams, E.S., Young, S. Chronic wasting disease of captive mule deer: a spongiform encephalopathy. *J Wildl Dis.* 1980. **16**, 89-98.

Wong, B.S., Chen, S.G., Colucci, M., Xie, Z., Pan, T., Liu, T., Li, R., Gambetti, P., Sy, M.S., Brown, D.R. Aberrant metal binding by prion protein in human prion disease. *J Neurochem.* 2001a. **78**, 1400-1408.

Wong, B.S., Liu, T., Li, R., Pan, T., Petersen, R.B., Smith, M.A., Gambetti, P., Perry, G., Manson, J.C., Brown, D.R., Sy, M.S. Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. *J Neurochem.* 2001b. **76**, 565-572.

Yam, A.Y., Gao, C.M., Wang, X., Wu, P., Peretz, D. The octarepeat region of the prion protein is conformationally altered in PrP(Sc). *PLoS One.* 2010. **5**, e9316.

Yao-yu, B.A.I., Ming-xing, J., Jia-an, C. Impacts of Environmental Factors on Degradation of Cry 1 Ab Insecticidal Protein in Leaf-Blade Powders of Transgenic Bt Rice. *Agricultural Sciences in China.* 2007. **6**, 167-174.

Yokoyama, T., Masujin, K., Schmerr, M.J., Shu, Y., Okada, H., Iwamaru, Y., Imamura, M., Matsuura, Y., Murayama, Y., Mohri, S. Intraspecies prion transmission results in selection of sheep scrapie strains. *PLoS One.* 2010. **5**, e15450.