THE *IN VITRO* CHARACTERISATION OF PRION DISEASES OF SHEEP

By

Maged M Taema BVM, MSc

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

November 2012

School of Veterinary Medicine and Science
Sutton Bonington Campus
Loughborough
Leicestershire
LE12 5RD

Author’s declaration:

I declare that, except where reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Nottingham or any other institution.

Signature: .....

Printed Name: Maged M Taema

Abstract:

One of the critical challenges in the transmissible spongiform encephalopathy (TSE) field is to understand the molecular basis of prion propagation and decipher the enigma of prion strains and their role in TSEs. The research approach adopted in this dissertation tackled different subjects of keen interest for prion characterisation and diagnostics.

A high throughput enzyme-linked immunosorbent assay (ELISA) was developed for detection of disease-related prion protein (PrP^{Sc}) using the protease thermolysin. Thermolysin allowed isolation of protease resistant PrP^{Sc} in its full-length form, while cellular prion protein (PrP^{C}) was digested. With further extraction and precipitation of PrP^{Sc} with sodium phosphotungstic acid (NaPTA) and in conjunction with using monoclonal antibodies that recognise distinct epitopes, PrP^{Sc} was detected and quantified successfully.

Molecular strain typing of ruminant TSEs was investigated using Western blotting and depending on the resistance of PrP^{Sc} to digestion with proteinase-K (PK) and thermolysin. The methods discriminated clearly between classical ovine scrapie and experimental ovine BSE. In contrast, experimental CH1641-like isolates showed heterogenous molecular profiles. In addition, the findings from this study demonstrated the existence of thermolysin-sensitive PrP isoforms which are resistant to PK and their presence varied between individual sheep and brain regions.

When studying prion propagation using the serial protein misfolding cyclic amplification (sPMCA) technique, different strains/isolates of ruminant prions were successfully amplified *in vitro* from as little as 0.01 ng of brain seed. Furthermore, ovine BSE was readily amplified *in vitro* in brain substrates from sheep with homozygous VRQ or AHQ *Prnp* genotype. In contrast, the CH1641 strain was refractory to such amplification. This method allowed for differentiation of experimental BSE from CH1641 prion strains within an ovine host, providing hope for the potential of sPMCA as a strain typing assay.

The use of bacterially expressed recombinant PrP^{sen} ($rPrP^{sen}$) as substrate in PMCA reactions (rPrP-PMCA) was assessed. The use of the substrate improved the sensitivity, specificity, practicality and speed of sPMCA assays for detecting a range of ovine prion isolates. Expression and purification of recombinant Syrian hamster prion protein (Sha rPrP) and VRQ ovine PrP (VRQ rPrP) provided substrate for detecting PrP^{Sc} in scrapie affected brain samples. Although both substrates had the same level of sensitivity, rSha PrP^{sen} had better specificity than VRQ rPrP. There were variations in amplification efficiency between different batches of the same rPrP.

This study recommends further investigations looking at the use of a range of experimental CH1641 and BSE samples, as well as using panels of CH1641-like field isolates for sPMCA reaction to establish (such) strain typing methodology. Furthermore, applying the rPrP-PMCA assay to detect PrP^{Sc} in secreta and excreta of infected sheep in the pre-clinical phase of the disease may provide a non invasive ante-mortem test for scrapie.

Publications:

The following papers contain work undertaken for this thesis:

MADDISON, B. C., OWEN, J. P., TAEMA, M. M., SHAW, G. & GOUGH, K. C. (2012): Temperature influences the interaction of ruminant PrP (TSE) with soil. *Prion*, 6.

Taema MM, Maddison BC, Thorne L, Bishop K, Owen J, Hunter N, Baker CA, Terry LA, Gough KC (2011): Differentiating Ovine BSE from CH1641 Scrapie by Serial Protein Misfolding Cyclic Amplification. Molecular Biotechnol, DOI:10.1007/s12033-011-9460-0

Maddison, B.C., Rees, H.C., Baker, C.A., Taema, M., Bellworthy, S.J., Thorne, L., Terry, L.A. and Gough, K.C., (2010): Prions are secreted into the oral cavity in sheep with preclinical scrapie. Journal of Infectious Diseases, 201(11): 1672-1676.

Gough KC, Baker CA, Taema M, Maddison BC (2009): In vitro amplification of prions from milk in the detection of subclinical infections. Prion. Vol. 3(4): 51-55.

Acknowledgements:

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

I would like to thank my supervisors, Dr Kevin Gough, The School of Veterinary Medicine and Science, University of Nottingham), Dr Ben Maddison (ADAS Biotechnology group, School of Veterinary Medicine and Science, University of Nottingham) and Dr Linda Terry (The Veterinary Laboratory Agency) for providing the scholarship, their scientific, personal and technical support.

I also would like to thank the staff of ADAS Biotechnology group; Dr Ben Maddison, Keith Bishop, Dr Jon Owen, Claire Baker, Helen Rees and Shila Patel who made my laboratory work as painless as possible, particularly during the first year of the study and their support till the end of the study.

I am also grateful and wish to express my deepest gratitude to my parents for their support and help throughout the whole of my life, and my wife and daughter for their support and help during this time.

Contents:

Table of contents:

List of Figures:

List of Graphs:

Chapter 3

List of Tables:

Chapter 1

Abbreviations:

°C	degrees Celsius
Bo	bovine
BSE	bovine spongiform encephalopathy
Cer	cerebellum
CJD	Creutzfeldt-Jakob Disease
CM	caudal medulla
CNS	central nervous system
CPG	deoxycytidyl- deoxyguanosine
CSSA	conformational solubility and stability assay
CWD	chronic wasting disease
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
fCJD	familial Creutzfeldt-Jakob disease
FFI	fatal familial insomnia
FPLC	fast protein liquid chromatography
FSE	feline spongiform encephalopathy
GdnHCl ½ values	the concentration of GdnHCl able to solubilise 50% of PrP^{Sc}
iCJD	iatrogenic Creutzfeldt-Jakob disease
g	gram
GALT	gut-associated lymphoid tissues
GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
Ha	hamster
HRP	horseradish peroxidase
Hu	human
IMAC	immobilised metal affinity chromatography
Immunohistochemistry or immunohistochemical	IHC
kDa	kilo dalton
LB	luria-bertani- agar
M	molar
mAb	monoclonal antibody

MBM	meat and bone meal
Mi	mink
ml	millilitre
mM	millimolar
Mo	mouse
ng	nanogram
OD	optical density
ODN	oligodeoxynucleotides
Ov	ovine
PBS	phosphate buffered saline
PBST	tween supplemented phosphate buffered saline
PK	proteinase K
PK PrP^{res}	PK resistant form of PrP
PK $rPrP^{res}$	PK resistant form of rPrP
PMCA	protein misfolding cyclic amplification
pNPP	p-nitrophenyl phosphate
Prnd	doppel protein gene (Dpl)
PRNP	prion protein gene in human
Prnp	prion protein gene in animals
Prnp $^{0/0}$ mouse	mouse lacking a functional prion gene at both alleles
PrP	prion protein
PrP^{C}	cellular (healthy) isoform of the prion protein
PrP^{d}	disease-specific prion protein
PrP^{res}	form of PrP relatively resistant to protease digestion
PrP^{Sc}	abnormal form of prion protein associated with genetic, sporadic and infectious forms of prion diseases.
PrP^{Sen}	collective term for PrP^{C} and PrP^{rec} (recombinant), sensitive to protease digestion, in contrast to PrP^{res}
rpm	revolutions per minute
rPrP	recombinant PrP
$rPrP^{res\ (Sc)}$	PK resistant fragments resulting from seeding rPrP substrate with PrP^{Sc} spike into PMCA reactions
$rPrP^{res(spon)}$	spontaneous (spon) PK-resistant fragments resulting from

	seeding rPrP substrate with healthy brain homogenates (PrP^{C}), or in non-seeded rPrP substrate in PMCA reactions
rSha PrP	recombinant syrian hamster PrP
sCJD	sporadic Creutzfeldt-Jakob Disease
SAF	scrapie associated fibrils
SC	spinal cord
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SHa	syrian hamster
SMA	powdered milk baby formula
SOD	superoxide dismutase
sPMCA	serial protein misfolding cyclic amplification
Sprn	shadoo protein gene (Sho)
sFI	sporadic fatal insomnia
TBS	tris-base
TH	thermolysin
TH PrP^{res}	TH resistant form of PrP
TME	transmissible mink encephalopathy
Tris	tris (hydroxymethyl) aminomethane
TSE	transmissible spongiform encephalopathy
µg	microgram
µl	microlitre
vCJD	variant Creutzfeldt-Jakob disease
VRQ rPrP	recombinant ovine VRQ PrP
V/V	volume per volume
w/v	weight per volume
g	gravitational force

Chapter 1: INTRODUCTION

1.1. Prion diseases:

Prion diseases are a group of hereditary, idiopathic and infectious diseases of humans and animals. These diseases are generally characterised by neurodegeneration and motor dysfunction due to pathological changes to the cellular isoform of the prion protein (PrP^{C}) and accumulation of a misfolded disease isoform of the prion protein (PrP^{Sc}). These diseases present a major threat to human health, as well as agricultural and wildlife animals (Tables 1.1 & 1.2).

Those prion diseases that have not been proven to be transmissible are sometimes referred to as prionopathies (Aguzzi *et al*., 2008). Within inherited human prion diseases, greater than 20 different mutations in the human prion protein (PrP) gene have been found to coincide with disease. However, such changes in the prion protein primary amino acid sequence are not a prerequisite for PrP^{Sc} formation, as mutations of the PrP gene are not found in sporadic forms of human prion disease that include the majority of CJD cases (Pruisner, 1997).

Transmissible spongiform encephalopathies (TSEs) are mainly a definition for prion diseases that result from exposure to infectious prion particles and can transmit between different hosts and species (Aguzzi *et al*., 2008, Pruisner 1997). Doherr, 2007 described TSEs as a group of human and animal diseases that share the following common features:

(a) Transmissibility between susceptible hosts either orally in nature or through other routes experimentally
(b) Long incubation periods without symptoms and symptoms only appearing in the final stage of disease
(c) Absence of immune response signs as an indication to disease pathology
(d) Spongiosis and vacuoles in specific brain regions are the main characteristic with histopathological lesions in the clinical phase of the disease
(e) Accumulation of amyloid plaques containing aggregates of the pathological isoform (PrP^{Sc}) of the physiological prion protein PrP^{C}
(f) No preventive vaccination or intervening therapeutic treatment are available

Table 1.1: Comparison of the epidemiological parameters of various TSE diseases of humans

Prion disease	Source of prions	References (first cited)
Human diseases		
Kuru	Infectious; exposure to contaminated human tissues during cannibalistic rituals	Gajdusek and Zigas 1957
Sporadic Creutzfeldt-Jakob disease (sCJD)	Spontaneous conversion of PrP^{C} to PrP^{Sc}	Creutzfeldt (1920) and Jakob (1921), cited by McKintosh *et al*., 2003
Familial Creutzfeldt-Jakob disease (fCJD)	Genetic; heritable mutations in the *PRNP* gene (Valine "V" or Methionine "M" at 129, and glutamic acid "E" or lysine "K" at 200. Another form of mutation where Valine "V" at 129 and aspartate "D" or asparagine "N" at 178).	Goldfarb *et al*., 1992
Iatrogenic Creutzfeldt-Jakob disease (iCJD)	Infectious; exposure to prion-infected surgical equipment, or tissue transplants; blood transfusion; human growth hormone therapy	Duffy *et al*., 1974
Variant Creutzfeldt-Jakob disease (vCJD)	Infectious; exposure to BSE-infected food including meat	Will *et al*. 1996
Gerstmann-Sträussler-Scheinker disease (GSS)	Genetic; heritable mutations in the *PRNP* gene	Gerstmann, 1928, cited by Mastrianni, 2004
Fatal familial insomnia (FFI)	Genetic; heritable mutations in the *PRNP* gene (where Methionine "M" at 129, and aspartate "D" or asparagine "N" at 178)	Roiter, 1974 cited by Cortelli *et al*., 1999
Sporadic fatal insomnia (sFI)	Unknown; somatic mutations or spontaneous conversion of PrP^{C} to the sFI confirmation of PrP^{Sc}	Mastrianni *et al*., 1999

Table 1.2: Comparison of the epidemiological parameters of various TSE diseases of animals

Prion diseases (host species)	Mechanism of transmission	References (first cited)
Scrapie (sheep & goat)	Infectious; ingestion or contact with scrapie-infected animals, tissues and secretions derived from the infected animals, or uptake from a prion contaminated environment	1723 cited by Deslys and Picot, 2002
Atypical scrapie/ Nor98 (sheep)	Unknown, spontaneous	Benestad *et al*., 2003
Transmissible mink encephalopathy (TME) (Mink)	Infectious; ingestion of prion-contaminated feed	Hadlow & Karstad, 1968
Bovine spongiform encephalopathy (BSE) (Cattle)	Infectious; ingestion of prion-contaminated feed	Wells *et al*., 1987
Atypical form of BSE in cattle (H-type) & (L-type)	unknown	Biacabe *et al*., 2004& Casalone *et al*., 2004
BSE in goat	unknown	Euro Surveill 2005;10(2):E050203.1
Feline spongiform encephalopathy (FSE) (domestic & wild cats)	Infectious; ingestion of BSE-contaminated feed	Aldhous, 1990, Willoughby *et al*., 1992, Baron *et al*., 1997
Spongiform encephalopathy in non-human primates	Ingestion of BSE-contaminated feed	Bons *et al*., 1999
Chronic wasting disease (CWD) (Deer, Elk, Moose)	Infectious; ingestion or contact with CWD-infected animals, tissues and secretions derived from the infected animals, or uptake from a prion contaminated environment	Williams *et al*., 1980 (first clinical cases had been seen in a captive (research) herd as early as 1965)
Exotic ungulate encephalopathy (Kudu, Oryx, Nyala, Eland, Gemsbok)	Infectious; foodborne exposure to BSE-infected tissue	Cunningham *et al*., 1993, Jeffrey and Wells, 1988, Kirkwood *et al*., 1990, 1993, 1994.

1.2. The prion protein: structure and function

The PrP protein is encoded by the prion protein gene (*Prnp*), and is 256 and 264 amino acids in length in sheep and cattle respectively. It has an amino-terminal signal peptide that directs the protein to the endoplasmic reticulum (ER) and that is cleaved shortly after translation. This is followed by an unstructured N-terminal domain containing an octapeptide repeat region, and a predominantly alpha-helical C-terminal domain with two variably utilised glycosylation sites. The C terminal region also contains a hydrophobic carboxy-terminal domain responsible for the attachment of the mature PrP^{C} protein to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor (Lloyd *et al*., 2011, Riesner, 2003, Pruisner *et al*., 1998).

Spectroscopic studies of hamster PrP^{C} have shown that PrP^{C} is rich in α-helices (42% α-helix content and little β-sheet content (approximately 3%)), whereas PrP^{Sc} has a high β-sheet content (43% β-sheet and 30% as α-helix) (Pan *et al*., 1993; Figure 1.1).

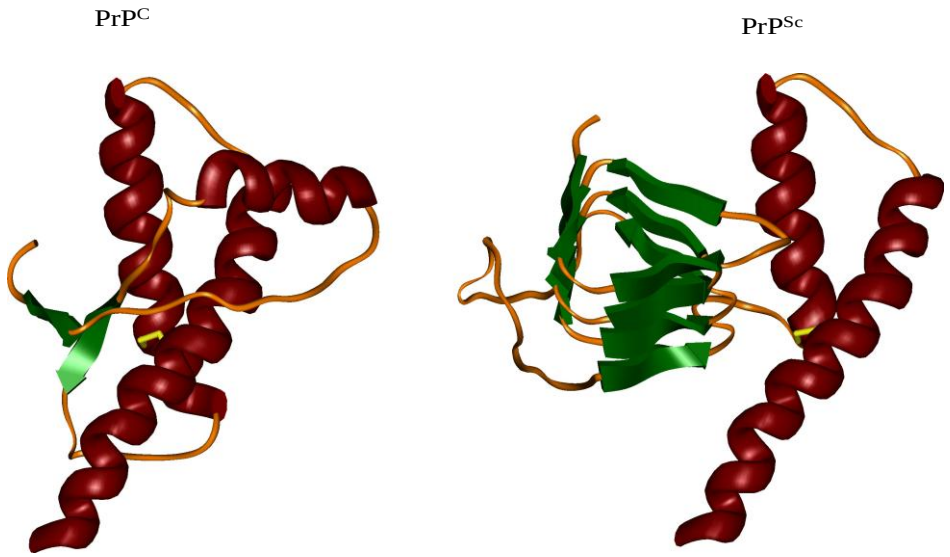


Figure 1.1: Schematic drawing to show the secondary structural features of the two isoforms of the prion protein, PrP^{C} with high α-helix content (red colour, left model) and PrP^{Sc} with a much higher β-sheet content (green colour, right model) (adapted from: http://www.cmpharm.ucsf.edu/cohen/)

PrP^C is a low abundance protein whose highest expression levels are found in nervous tissues within neuronal and glial cells (Aguzzi *et al*., 2008, Prusiner *et al*., 1998). It is also expressed at lower levels by many other cells and various tissues, such as blood, digestive tract, lymphocytes, and skin (Bendheim *et al*., 1992). It is known that PrP^C is an absolute requirement for the development of a prion disease as studies have shown that PrP-gene deficient mice were resistant to scrapie and did not develop ataxia or sever pathologies after challenge with infectious inoculum (Bueler *et al*., 1992).

One of the challenges in prion biology is determining the main physiological functions of the cellular prion protein. Research reports have linked its functional role to different biological processes such as copper binding (Brown *et al*., 1997), copper internalization and cell haemostasis (Kretzschmar *et al*., 2000), neuronal sensitivity to oxidative stress through regulating superoxide dismutase-1 (SOD-1) activity in a copper-dependent manner (Brown *et al*., 2002). Furthermore, PrP^C mediates cellular iron uptake and transport not only in cell culture (Singh *et al*., 2009), but mice lacking PrP^C expression ($Prnp^{0/0}$) recover slowly from experimentally induced haemolytic anaemia (Zivny *et al*., 2008). Moreover, PrP^C is involved in the process of intestinal epithelial cell proliferation and in the sub-cellular distribution of proteins involved in cell architecture and junctions (Morel *et al*., 2008). In addition, other research reports suggested that PrP^C has a neuroprotective function against internal or environmental stresses and regulating apoptosis (Roucou and LeBlanc 2005 and Roucou *et al*. 2004). Also, due to the localisation of PrP^C in the lipid raft domains on the plasma membrane, it has been suggested that PrP^C can activate transmembrane signalling pathways through interaction of PrP^C with specific protein or peptide ligands and initiating signal transduction (Westergard *et al*., 2007, Kanaani *et al*., 2005, Santuccione *et al*., 2005, Chen *et al*., 2003). Recently, a preliminary study using mouse brain cell lines has shown that PrP^C might be necessary for brain microvascular endothelial cells to migrate into damaged regions in the brain (Watanabe *et al*., 2011). However, to date there is no definitive consensus as to the function of this protein.

1.3. Aetiology:

For decades, the molecular structure of the scrapie agent was the most puzzling subject for prion scientists. The transmissibility, long incubation period and existence of many different strains, have led to the hypothesis that it is a "slow virus" (Hadlow, 1959) or "viroid" or spherical infectious particle of approximately 25 nm that could accommodate a genome of 1-4 kb in length and probably encodes its own protective nucleocapsid (Manuelidis *et al*., 2009, Manuelidis, 2007, Manuelidis *et al*., 2007). Various trials to elucidate its nature, based on the usual known characteristics of infectious agents, have failed to isolate any nucleic acid, and the agent retains its infectivity during many physical and chemical treatments that would kill conventional infectious agents such as bacteria, viruses and fungi. Further research provided clear evidence that the scrapie agent is a hydrophobic form of PrP^{Sc} and the word "prion" was adopted which originated from "proteinaceous infectious particles". The prion is thought to be a protein capable of propagation without the need for nucleic acid (Prusiner 1997, 1991 & 1982), the so called "protein-only" hypothesis. Recently, the generation of a classic neuropathology of prion disease through intracerebral injection of aggregated, protease-resistant bacterially expressed recombinant prion protein (rPrP) in wild-type mice has supported the protein-only hypothesis (Wang *et al*., 2011, Wang *et al*., 2010).

According to the protein-only hypothesis, the central biochemical event occurring during TSE pathology is PrP^{Sc} self-replicating by interacting with PrP^{C} and inducing its structural conversion to PrP^{Sc} (Prusiner 1998 & 1991). The infectious prion proteins denoted as PrP^{Sc} and/ or PrP^{res} (protease-resistant core of the prion protein), have the same amino acid sequence as PrP^{C} but a different three dimensional structure. The conversion process is not fully understood. In view of the protein-only model proposed by Prusiner, the transition of PrP^{C} to PrP^{Sc}, results from the interaction between normal (healthy) and an infectious prion molecule to form a PrP^{C}–PrP^{Sc} heterodimer and subsequently, restructure of the normal PrP^{C} molecule into PrP^{Sc}. Consequently, after separation of the two molecules, the two new PrP^{Sc} molecules can again bind healthy PrP^{C} molecules and convert it to PrP^{Sc}. This autocatalytic conversion process results in crucial changes to the prion protein from its predominantly α-helical structure to a β-sheet rich insoluble misfolded form that forms oligomers and is partially resistant to proteases including proteinase-K (PK).

This hypothesis was strengthened by the development of a variety of cell-free reactions for prion conversion and propagation. These reactions have shown that the conversion of PrP^{C} into a protease resistant PrP^{Sc} occurres via a seeded or templated polymerization mechanism (Castilla *et al*., 2006, Horiuchi *et al*., 2000 & 1999, Caughey *et al*., 1995, Jarrett and Lansbury, 1993, Gajdusek, 1988).

1.4. Epidemiology:

1.4.1. Disease transmission:

Different routes of transmission were found to exist for different TSE strains and host species. Doherr, 2007, has described a general base for TSEs main transmission routes (Figure 1.2). The earliest record for TSE diseases was for sheep scrapie in Europe in 1732 (Deslys and Picot, 2002). After that, it has been reported in many countries with exceptions including Australia and New Zealand. As the prototypic prion disease, scrapie in sheep became one of the most studied TSEs and has facilitated our understanding of these diseases. Sheep susceptibility to scrapie was at first thought to be heritable (Parry, 1962). Further studies looked to the potential of breed effect on the susceptibility to infection, but it was revealed unlikely and it became clear that there was much variation in the susceptibility between individual animals within breeds (Davies and Kimberlin 1985, Nussbaum *et al*., 1975, Pattison, 1974, Dickinson *et al*., 1968). After more extensive experimental challenge of different breeds with scrapie, it became obvious that genetics was playing a significant role in scrapie susceptibility and in particular a single gene known as Sinc (referred to scrapie incubation time), a gene that was defined later as the *Prnp* gene coding for the so-called prion protein, PrP^{C} (Hunter *et al*., 1996, Hunter *et al*., 1993, Goldmann *et al*., 1991, Pruisner 1982). Later, it was revealed that polymorphism of the *Prnp* gene of a host has a profound impact on the susceptibility and resistance to prion diseases.

It is now evident that the mode of transmission of TSEs is mainly through (a) horizontal transmission: exposure to infectious prion particles via the oral route through ingestion of contaminated food or contact with infected materials in the environment and gaining entry to the body through the gut-associated lymphoid tissues in Peyer's patches in the alimentary tract and (b) vertical transmission: when

infection occurs between affected mothers and their offspring in certain animal species.

Since the discovery of BSE in UK herds in 1986 (Wells *et al*., 1987), and establishing a possible link between BSE in cattle and other species (Kirkwood *et al*., 1994 & 1993, Cunningham *et al*., 1993, Aldhous, 1990, Kirkwood *et al*., 1990, Jeffrey and Wells, 1988), particularly to humans as the main cause of vCJD (Bruce *et al*., 1997), major research efforts have been intensified to illucidate the nature and origin of BSE.

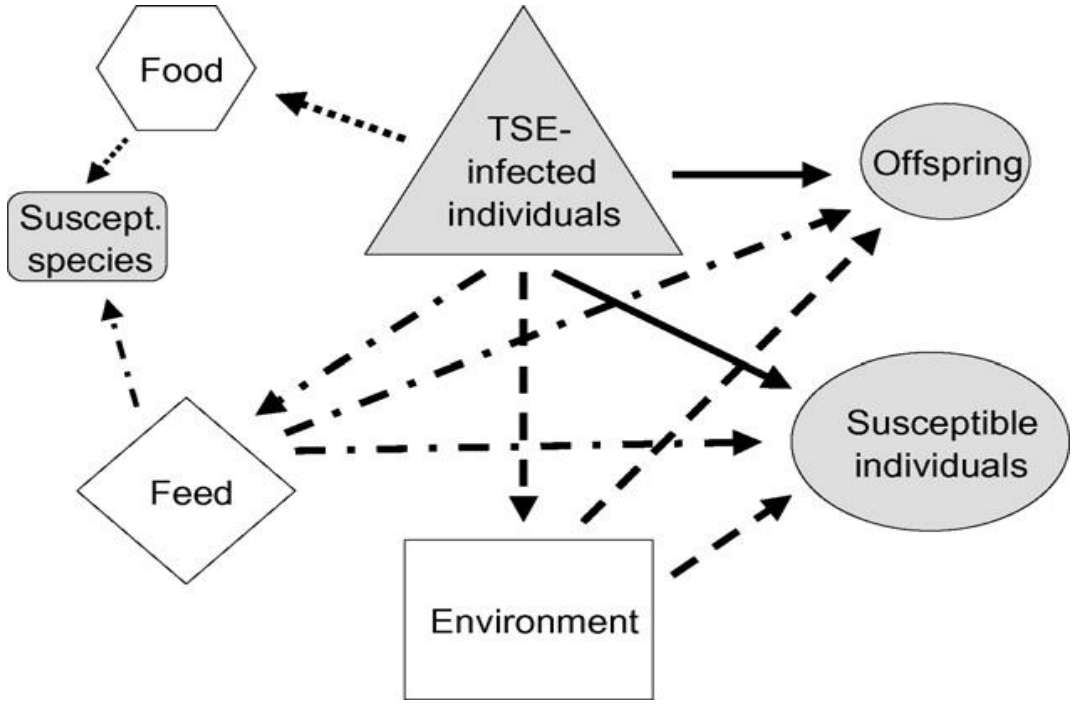


Figure 1.2: Main transmission routes for transmissible spongiform encephalopathy (depending on TSE strain and host species). Solid lines: direct vertical (intra-uterine, offspring) and horizontal (direct contact) transmission. Dashed lines: indirect horizontal transmission via environment (excretion, uptake), which may be most relevant for scrapie and CWD. Dash-dotted lines: indirect horizontal transmission via feed (recycling) which may be most relevant for BSE and related diseases. Dotted lines: indirect horizontal transmission via food which may be most relevant for vCJD (BSE contaminated beef) and Kuru (cannibalistic burial ceremonies) (adapted from Doherr, 2007).

Different hypotheses were proposed for the origin of the BSE prion that include: (i) that one of the sheep or goat scrapie strains was infectious for cattle. This remains yet to be proven (ii) that there might be a spontaneous somatic mutation in the *Prnp* gene, or spontaneous conversion of PrP^{C} to PrP^{Sc} similar to sporadic CJD in humans, that could have produced a TSE strain either in sheep or in cattle that was infectious for cattle (Doherr, 2007).

It is believed that the BSE epidemic resulted from the oral route of transmission with meat and bone meal (MBM) containing bovine prion being fed to dairy cows. However the origin of the BSE prion has not been determined (Prusiner 1997, Wilesmith, 1991).

The identification of a BSE case associated with the novel mutation E211K (glutamic acid 211 lysine) within the prion protein gene (*Prnp*), similar to the pathogenic mutation E200K in humans that has been described as the most common cause of genetic CJD has supported this view (Richt and Hall, 2008). Biochemical, neuropathological and transgenic animal experimental studies suggest that BSE has transmitted to humans causing vCJD (Bruce *et al*., 1997, Hill *et al*., 1997), while there is no such evidence that classical scrapie is linked to any of the known diseases in human.

Molecular (strain typing) and epidemiological findings showed the ability of BSE to cross species barriers. This was demonstrated in the detection of feline spongiform encephalopathy (FSE) in domestic & captive wild cats (Baron *et al*., 1997, Willoughby *et al*., 1992, Aldhous, 1990). In addition, different captive herbivore animals (Kudu, Oryx, Nyala, Eland, Gemsbok) were diagnosed with spongiform encephalopathy (Kirkwood *et al*., 1994 & 1993, Cunningham *et al*., 1993, Jeffrey and Wells, 1988, Kirkwood *et al*., 1990). Furthermore, the susceptibility of small ruminants to BSE has caused a great concern that BSE infected small ruminants could present a zoonotic risk to human through consumption of food products derived from small ruminants. Experimentally, BSE can be transmitted to sheep and between sheep in a closed flock (Bellworthy *et al*., 2005), however so far, no field cases of BSE in sheep have been diagnosed. In contrast, a limited number of BSE cases in goat have been diagnosed (Spiropoulos *et al*., 2011, Jeffrey *et al*., 2006b, Eloit *et al*., 2005), raising concern for a public health risk.

In CWD of captive and free ranging deer and moose, the mode of transmission is mainly through the horizontal route, particularly through contamination of the

environment with secreted and excreted biological materials (Gilch *et al*., 2011). In addition, experimental challenge of meadow voles (*Microtus pennsylvanicus*), red-backed voles (*Myodes gapperi*) and deer mice (*Peromyscus maniculatus*) highlighted the potential role of scavenging rodents to act as reservoir for CWD infection (Heisey *et al*., 2010). However, although cross species transmission of CWD to other ruminant species was not proved to occur naturally, experimental transmissions to sheep, goat and cattle through intracerebral inoculation were successful to transmit the infection but appeared to be of low efficiency (Hamir *et al*., 2006, Hamir *et al*., 1993, Williams *et al*., 1992). Importantly, there is no evidence that CWD has transmitted to humans, and epidemiological and experimental studies classified the risk as low zoonotic potential (Saunders *et al*., 2012, Gilch *et al*., 2011).

1.4.2. Factors contributing to host resistance and susceptibility to TSE diseases:

1.4.2.1. Genotypes and Prnp gene polymorphism:

Many studies have shown that sheep resistance or susceptibility to scrapie is dependent upon the genotype of the host and strongly linked to the single nucleotide polymorphic mutations at codons 136, 154 and 171 out of the 256 amino acids of the *Prnp* gene. Mutations linked to disease susceptibility were either arginine (R), glutamine (Q) or histidine (H) at codon 171; histidine (H) or arginine at codon 154, and valine (V) or alanine (A) at codon 136. These complex polymorphic mutations have produced sheep with different genotypes; approximately 15 genotypes with five common alleles: $A_{136}R_{154}R_{171}$, ARQ, ARH, AHQ and VRQ, that differ widely in their degree of resistance and susceptibility to scrapie (Baylis and Bilheude *et al*., 2007, Goldmann, 2004, Hunter *et al*., 1996, Belt *et al*., 1995, Clouscard *et al*., 1995). These genotypes and their related length of incubation period or apparent resistance to infection have informed breeding programmes to produce flocks free of scrapie and/or minimising disease incidence. Such breeding programmes have been employed in the UK and other European countries. In addition, many reports have shown polymorphism in other codons that have been associated with sheep resistance to infection. Recently, T^{112}ARQ homozygous sheep where a single nucleotide polymorphism (SNP) locus at codon 112 resulted in threonine instead of methionine (M112T) has been linked to more resistance to the development of classical scrapie

(Laegreid *et al*., 2008). Further SNPs of the sheep *Prnp* have been reported, such as G127V/S, L141F, H143R, M157I, Q171H/R/K, Q189L, Q220H, and R223K, but more investigations are needed to understand their relationship to scrapie susceptibility or resistance (Wang *et al*., 2008, Billinis *et al*., 2004, Thorgeirsdottir *et al*., 1999). At the same time, the incidence rate of these SNPs is variable within flocks, breeds, and countries. Within all ruminant species, the significance of SNP and genotypes could not be estimated as there are at least 105 alleles that encode 85 distinctive mature PrP protein variants. These variable genotypes may reduce the potential of TSE incidence and may result in unsuccessful or inefficient transmission within and between species (Goldmann, 2008). Therefore, it is difficult to speculate on the ability of a particular strain of scrapie to infect different host species (Cancellotti *et al*., 2007).

1.4.2.2. Role of other genes:

Although, the polymorphism of *Prnp* gene is mostly influencing prion disease susceptibility and pathogenesis, the role of other genes has been suggested to be associated with prion disease progression. One homologues gene *Sprn* (encoding shadoo protein, Sho) that is expressed in the central nervous system (CNS), has shown a positive expression correlation with PrP^{C} in the cerebral and cerebellar cortex of sheep (Lampo *et al*., 2009). This positive correlation might indicate co-regulation between these genes and consequently an influence on prion disease progression (Gossner *et al*., 2009, Stewart *et al*., 2009, Watts *et al*., 2007); the authors suggested that Sho protein may have a PrP^{C}-like neuroprotective activity. Further studies are of considerable importance to establish such association and its impact on prion diseases in animals. A second homologues gene to *Prnp*, is *Prnd* (encoding doppel protein, Dpl) but this has little known role in the mechanism of prion disease pathology (Gossner *et al*., 2009).

The *in vivo* manipulation of 3 genes: mainly ablation of the amyloid beta (A4) precursor protein (APP), or interleukin-1 receptor, type I (IL-1R1), and transgenic overexpression of human SOD-1, have all been linked to prolonged prion disease incubation period in inbred mice. However these observations have been difficult to interpret due to inter-experimental variations (Tamguney *et al*, 2008).

1.4.2.3. Toll-like receptors (TLRs):

The role of the toll-like receptors (TLRs) family and its agonists in activating the host innate immune response against various pathogens including prions has been the central point of many immunological research studies (Spinner *et al*., 2008, Prinz *et al*., 2003). Two research studies have shown that injection of deoxycytidyl-deoxyguanosine (CpG) oligodeoxynucleotides (ODN) enhanced the humoral immune response to prions via TLR9 and also the production of antibodies to prion protein (Spinner *et al*., 2007). Spinner *et al*., 2008 also suggested that TLR4 activation interferes with scrapie pathogenesis in transgenic mice. All of these studies aimed to provide an alternative model for developing preventive or therapeutic drugs to prion diseases in humans, and whilst identifying TLRs as potential therapeutic targets, their role in prion pathogenesis is yet to be fully understood.

1.5. Clinical signs:

In humans, prions could persist silently for nearly a third of a host's life span before being detectable. The clinical symptoms of human prion diseases are variable between different forms of the disease; the genetic polymorphism of the *PRNP* gene, the age of onset and duration of disease and the family medical history are greatly influencing the clinical phenotype of prion diseases in human (Head and Ironside, 2012). At terminal stage, ataxia and rapid cognitive decline causing dementia are the main symptoms of prion disease, ending in death (Aguzzi *et al*., 2008). In sheep scrapie, clinical signs are variable; these can include unusual social behaviour and extreme nervous reactions to stimuli such as human contact, ataxia, change in the fleece colour, pruritis due to scratching against fence posts and biting the affected area usually around the base of the tail and occasionally the whole of the side of the body. Animals lose the ability to feed themselves and muscle wasting may occur at the final stage of the disease. In experimentally BSE-infected sheep, the illness is more acute with shorter duration (Hunter *et al*., 2003). However, variations within breeds were reported.

In BSE affected cattle, the signs included: abnormal gait, particularly ataxia of the hind limbs; tremors and abnormal behaviour including nervousness or aggressiveness;

jumping up suddenly from a lying position and hyper reactivity to stimuli (Konold *et al*., 2004).

In CWD of cervids, the most common characteristic sign is gradual decrease in appetite and weight loss, behavioural alterations gradually arise (such as loss of fear of humans, disorientation, and intermittent circling to one side), together with a lowered head and drooping ears. In a more clinically progressed stage, affected animals show excessive salivation and difficulty in swallowing, regurgitation of food and ruminal fluid, ruminal atony, and polyuria. In a later stage of the disease, the animal becomes recumbent and might develop aspiration pneumonia (Imran and Mahmood, 2011, Hamir *et al*., 2008, Sigurdson and Miller, 2003).

1.6. Disease pathology:

In ovine scrapie, CWD and ovine BSE, once an animal has contracted the infectous prion by the oral route, accumulation and propagation of PrP^{Sc} occur in the gut-associated lymph nodes. This is followed by general progression and widespread involvement of other lymphatic tissues: palatine tonsils, retropharyngeal and mesenteric lymph nodes, Peyer's patches, and spleen (van Keulen *et al*., 2008, Andreoletti *et al*., 2000, Heggebo *et al*., 2000, van Keulen *et al*., 1996). This prolonged widespread replication of PrP^{Sc} in the lymphatic tissues, and hematogenous dissemination in blood, extends to the central and peripheral nervous system where it progressively accumulates, causing fatal neurodegenerative changes. Also, it is worth noting that a lack of PrP^{Sc} in lymphoid tissues from sheep with different genotypes and from different breeds (Ligios *et al*., 2006, Houston *et al*., 2002) as well as, in cattle BSE (Beekes *et al*., 2008, Wells *et al*., 2005, Terry *et al*., 2003) was also recorded, yet PrP^{Sc} accumulation within the CNS was still apparent.

Spongiosis and vacuolation within the brain are the most consistent characteristic lesion profiles of TSEs and such changes are visible under the light microscope as holes in the tissue sections. In experimentally infected sheep, the severity and neuroanatomical distribution of vacuolations and neuronal loss are features indicative of the strain of scrapie with which the animal has been infected. The lesion profile may also be influenced by route of infection, age at infection, the duration of clinical signs, and sheep genotype (Hunter *et al*., 2003, Begara-McGorum *et al*., 2002, Ligios *et al*., 2002). At the same time, breed of sheep or age at death (Wood *et al*., 1997)

and the strain agent (Begara-McGorum *et al*., 2002) were reported to contribute to the variation in the pathological lesions in natural sheep scrapie. In experimentally-infected mice with BSE, a mild to moderate vacuolation in the grey matter of the hypothalamus, medulla oblongata and septum, and a more severe vacuolation of the cochlear nucleus was the main characteristic lesion that is similar to vCJD and different from sCJD and scrapie strains. This strain-dependent characteristic pathological lesion profile was one of the lines of evidence that indicated humans had been infected with BSE contaminated meat as the reason for vCJD (Bruce *et al*., 1997).

1.7. Atypical prion diseases in animals:

1.7.1. Atypical scrapie:

Atypical scrapie is a sheep TSE designated Nor98, having been diagnosed first in Norway. It differs from classical scrapie in the neuroanatomical distribution of the histopathological lesions and PrP^{Sc} deposition in the brain; with predominance of pathological lesions in the cerebellar and cerebral cortices and not in the brain stem as in classical scrapie (Benestad *et al*., 2003). When analysed by Western blot, the associated PrP^{Sc} is less PK-resistant than that found in classical scrapie and also characterised by a distinct banding pattern and molecular weight profile for protease-resistant PrP^{Sc} fragments compared to classical scrapie (Buschmann *et al*., 2004). Atypical scrapie is also distinct from classical scrapie in the relationship between susceptibility to disease and *Prnp* polymorphisms; as it occurred in sheep whose genotype is associated with resistance to classical scrapie and has also been found in closed flocks of sheep (Buschmann *et al*., 2004). The aetiology, epidemiology, source and risk of atypical scrapie are difficult to explain. However, in different random surveys, they found that a polymorphism at amino acid 141 (Phenylalanine/Leucine) (AF141RQ allele or AL141RQ), is linked to susceptibility to atypical scrapie where the majority of atypical scrapie cases were ALHQ/ALHQ, ALHQ/AFRQ, and AFRQ/AFRQ while ALHQ/ALRQ and AFRQ/ALRQ were less susceptible. Furthermore, most cases contained the AHQ allele and also several sheep with ARR/ARR genotype were diagnosed with atypical scrapie (Tranulis *et al*., 2011, Luhken *et al*., 2007, Moreno *et al*., 2007, Saunders *et al*., 2006). It has been speculated that atypical scrapie may be a spontaneous prion disease analogous to

sCJD in humans (Benestad *et al*., 2003). In the UK, the earliest records points to sheep dying of atypical scrapie in 1987 (Webb *et al*., 2009) and 1989 (Hunter, 2007).

1.7.2. Atypical BSE:

Since 2004, BSE cases with distinct features from classical BSE ("BSE-C" disease) were reported in different countries and were called atypical BSE. (Stack *et al*., 2009, Gavier-Widén *et al*., 2008, Jacobs *et al*., 2007, Buschmann *et al*., 2006, Biacabe *et al*., 2004, Casalone *et al*., 2004). Analysis of some cases of these unique infections by Western blot showed an unusually high molecular mass of PrP^{Sc} compared to BSE-C and such cases were called H-type BSE (Stack *et al*., 2009, Gavier-Widén, *et al*., 2008, Biacabe *et al*., 2004). In other cases, the PrP^{Sc} molecular mass was slightly lower than that observed in BSE-C, together with a lower proportion of the diglycosylated band and these were termed L-type BSE (Stack *et al*., 2009, Baron and Biacabe, 2006, Casalone *et al*., 2004). Both H-type and L-type BSE have PrP^{Sc} with enhanced protease sensitivities at pH 8 (Jacobs *et al*., 2007). The L-type atypical BSE is characterised by the presence of amyloid plaques which are not observed in BSE-C. Therefore, the disease was also termed Bovine Amyloidotic Spongiform Encephalopathy (BASE). In addition, the neuro-anatomical lesions are more abundant in the cortical regions in contrast to the brain stem for cases of BSE-C (Casalone *et al*., 2004).

1.8. Diagnosis of TSEs:

1.8.1. Post-mortem diagnoses:

Since the detection of the first case of BSE in cattle in 1986 (Wells *et al*. 1987) and the subsequent discovery of the potential for cattle BSE to be transmitted to humans and cause vCJD in 1996 (Brown *et al*., 2001, Bruce *et al*., 1997), there was an urgent need for the development of rapid tests for post-mortem diagnosis of TSEs in order to screen food chain animals to reduce the exposure of the human population to the BSE agent. Prior to this, post-mortem histological and immunohistochemical (IHC) techniques showing typical TSE characteristic lesions (spongiosis, amyloid plaques) in the brain was the main confirmatory tool to diagnose suspected clinical cases (Fraser, 1976). With more understanding of the pathogenesis of TSEs and lymphatic/haematogenic dissemination of amyloids, the IHC technique was extended

to examine samples of tonsils, Peyer's patches, and other gut-associated lymphoid tissues in scrapie affected sheep (van Keulen *et al*., 2008, Andreoletti *et al*., 2000, van Keulen *et al*., 1996). In addition, exploiting the biochemical features of PrP^{Sc} towards protease digestion formed the basis to differentiate between PrP^{C} and PrP^{Sc} in more rapid diagnostic tests. PrP^{C} is susceptible to PK digestion and PrP^{Sc} is partially resistant. Treating prion infected samples with PK results in full digestion of PrP^{C}, and the retention of N-terminally truncated PrP^{Sc} conformers. Therefore, susceptibility and resistance of PrP to PK digestion and identification of a protease resistant C-terminal region is a widely-used tool to diagnose prion diseases. Furthermore, Owen *et al*., 2007, found that treating PrP^{Sc} infected tissue with a distinct protease, thermolysin (TH), reveals a full length PrP^{Sc} that is resistant to proteolysis, and digests PrP^{C} into small protein fragments. Again, providing the diagnostic tools to reveal the presence of PrP^{Sc} within biological samples.

Western blot techniques have been used to detect the PK resistant (PK PrP^{res}) C-terminal regions, which remain intact after limited protease digestion with PK. Detection of this protease resistant 'core' is dependent on antibodies that bind to an epitope situated in the C-terminal region. This technique has been one of the main tools in the diagnosis of TSEs and characterisation of prion strains. Strain discrimination is based on the molecular weights and glycoform ratios produced by the three glycoforms of PrP^{Sc} such that Western blot patterns can be found that are unique to individual prion strains and are often stable between host species (Stack *et al*., 2006, Stack *et al*., 2002, Prusiner 1998). Furthermore, different analogous immunodetection techniques have been described for the detection of PrP^{Sc} aggregates in biological samples such as dot blots, and various enzyme-linked immunosorbent assays (ELISAs) (Creminon *et al*., 2004) including time-resolved fluorescence (TRF)- DELFIA (dissociation enhanced Lanthanide fluoroimmunoassay) enhanced detection assays (Barnard *et al*., 2000) and conformation dependent immunoassay (CDI) (Thackray *et al*., 2007, McCutcheon *et al*., 2005, Safar *et al*., 2005, Safar *et al*., 2002, Bellon *et al*., 2003, Safar and Prusiner, 1998, Safar *et al*., 1998).

The DELFIA detection system is based on the previously reported ELISA-format but with the advantage of using fluorescent chelates. Therefore, the signal is developed in a few minutes and is stable up to hours. In addition, Barnard *et al*., 2000, exploited the solubility of PrP isoforms in two concentrations of guanidine hydrochloride

without the need for proteolysis to discriminate between PrP^{C} (soluble isoform) and PrP^{Sc} (less soluble disease associated aggregated isoform). Following quantification of the two isoforms, the quantification of disease-associated PrP as a percentage of total PrP becomes a measurable parameter that identified healthy from infected animals.

In the CDI, the technique relies on the differential binding of antibodies to native or denatured PrP^{Sc} rather than resistance or sensitivity to PK. In summary, a specific antibody will bind to an exposed epitope in native PrP^{C} while the same epitope is buried in non denatured native PrP^{Sc}. After denaturation, this epitope will become exposed in PrP^{Sc} and is then detected by the antibody. The differences in binding leads to quantifiable differences between the signal ratio given by denatured and native PrP^{Sc} compared to the ratio of signals for denatured/native PrP^{C} (Thackray *et al*., 2007, McCutcheon *et al*., 2005, Safar *et al*., 2005, 2002 & 1998).

Experimental animals, particularly rodents, have been thoroughly exploited as the most sensitive and specific method of diagnosing and studying the pathogenesis of prion diseases. After experimental infection of an animal with the suspected infected biological material, animals are usually monitored for the advent of clinical disease. TSE-disease is then confirmed through post-mortem detection of PrP^{Sc} and characteristic brain lesions through histology, IHC and Western blot analysis (Grassi *et al*., 2008). However, the application of rodent bioassay as a routine screening tool for TSE diseases is not feasible due to ethical considerations and because the method takes several months to years to complete, as the onset of clinical signs and the accumulation of detectable amounts of PrP^{Sc} can be very slow during the incubation phase (Castilla *et al*., 2006). Susceptibility to prion disease and the length of incubation periods before symptom onset differs between species and can change dramatically when infectious prion strains are transmitted between species (Sweeting *et al*., 2010) leading to the "species barrier" phenomenon. Experimental studies showed that transmission of prion to a new species is less efficient than intra-species transmission (Béringue *et al*., 2008). However, the transmission may result in efficient propagation and infection (Konold *et al*., 2006), probably after a few sub-passages and adaptation of the infectious prion to the new host (Béringue *et al*., 2008) or it might fail to cause disease (Kimberlin *et al*., 1978) reflecting an intrinsic resistance of the host to the disease. Therefore, transgenic animal models where mice carry the *PRNP* gene of other species can be useful in evaluating the transmissibility

and pathogenesis of prion diseases. Taken all together, combination of factors such as prion strain, transgenic/wild type animal line, dose and route of challenge dictate the incubation time before clinical onset of disease (Béringue *et al*, 2008).

Different analytical methods and diagnostic tests have been developed for routine TSE diagnosis based upon the immunological detection of PrP^{Sc} (Grassi *et al*., 2008). The European Directorate General for Health and Consumer Protection has evaluated and approved different commercial, highly sensitive and accurate diagnostic tests that analyse tissues sampled post mortem for large-scale epidemiological studies identifying animals infected by TSEs. Such widespread surveillance is undertaken to minimise human exposure to prion agents through animal products. Such steps became of great importance following the BSE-epidemic within the UK and subsequent expanding of the BSE geographical range to other European countries. The evaluation of these tests within a series of studies proved that the TSE Kit (Enfer), Prionics-Check LIA and Prionics-Check Western (Prionics), and CEA (Saclay, France) tests have 100% sensitivity and specificity. In addition, TeSeE and TeSeE sheep/goat (Bio-Rad), TSE post-mortem test (IDEXX), Prionics-Check Western SR and Prionics-Check LIA SR (both from Prionics) had the advantage of detecting atypical scrapie in brain stem samples.

Although all of these tests have contributed significantly to the diagnosis of prion diseases and protecting humans from consuming contaminated meat, their application is still limited to post-mortem examination of specific nervous tissue samples.

1.8.2. Ante-mortem diagnosis of scrapie in sheep:

Recent studies of prion pathogenesis, dissemination and accumulation of PrP^{Sc} in infected animals together with IHC detection, CDI-ELISA, cell free conversion assays and animal bioassays have shown widespread distribution of PrP^{Sc} in a wide range of different tissues and excreta/secreta during the clinical and pre-clinical phase of scrapie (Table 3.1).

The understanding of the pathogenesis of scrapie and in particular the early lymphatic/haematogenic dissemination and detection of PrP^{Sc} from Peyer's patches of the intestine to progressive spread into other lymphoid organs (lymph nodes, spleen) has facilitated the development of ante-mortem tests for scrapie. One such

ovine scrapie test was based on the IHC examination of biopsies from the third eyelid (O'Rourke *et al*., 2002, 2000 & 1998). Then, the methodology was applied further to pharyngeal tonsils and rectal mucosa (Gonzalez *et al*., 2008b), and allowed the preclinical diagnosis of disease. Although this methodology had potential in confirming suspected cases, the sensitivity of these tests has varied considerably between different studies (Monleon *et al*., 2011, Gonzalez *et al*., 2008b, O'Rourke *et al*., 2002). Furthermore, the frequency of positive results was affected by age or incubation in both natural and experimental infection. It has been shown that its application might be hindered by low numbers of lymphoid follicles and PrP^{Sc} accumulation in the biopsy materials. Moreover, the degree of involvement of lymphoid tissue in scrapie pathogenesis is influenced by the host genetic code and in turn, the availability and level of detectable PrP^{Sc} in the biopsied lymphoid tissue particularly in ARQ/ARQ sheep (Monleon *et al*., 2011).

Taking into account the routes of prion transmission (Mathiason *et al*., 2009, Doherr, 2007, Safar *et al*., 2008) particularly with CWD and scrapie, there might be potential for environmental contamination and lateral or indirect horizontal transmission of the disease between flock- or herd-mates as well as exposure of other species to prions. Such environmental dissemination of infectivity through biological secretion and excreta is likely to be from both clinical and subclinical carrier animals. Further animal studies have demonstrated that oral exposure to prion infected blood, urine, saliva, faeces in the animal environment; exposure to infected environmental fomites or excreta from subclinical infected animals can be enough for prion transmission in CWD or scrapie (Maddison *et al*., 2010, Mathiason *et al*., 2009, Safar *et al*., 2008, Mathiason *et al*., 2006). Based on these findings, a considerable research effort is still in progress to develop further ante-mortem, pre-clinical diagnostic tests for prion diseases using readily accessible biological samples. Such tests would contribute to the eradication of pre-clinically infected animal and therefore control of the unseen transmission pathways of prion diseases.

Table 1.3: Detection of prion infectivity or PrP^{Sc} within excreta / secreta or within tissues.

Biological material	Prion strain/ host species	Prion detection method	Disease status	Reference
Mammary gland	scrapie/sheep	Western blots, IHC	clinical	Ligios *et al*., 2005
Milk	scrapie/sheep	Ovine bioassay	clinical	Konold *et al*., 2008
	scrapie/sheep	rodent bioassay	preclinical/clinical	Lacroux *et al*., 2008
	scrapie/sheep	sPMCA[(a)]	preclinical/clinical	Maddison *et al*., 2009
Faeces	scrapie/hamster	CDI	preclinical/clinical	Safar *et al*., 2008
	scrapie/hamster	sPMCA	clinical	Krüger *et al*., 2009
	scrapie/sheep	sPMCA	preclinical/clinical	Terry *et al*., 2011
	CWD/deer	Cervid bioassay	preclinical/clinical	Tamgüney *et al*., 2009
Urine	scrapie/mouse	rodent bioassay	preclinical/clinical	Seeger *et al*., 2005
	scrapie/hamster	sPMCA	clinical	Murayama *et al*., 2007
	scrapie/hamster	rodent bioassay	clinical	Gregori *et al*., 2008
	scrapie/hamster	sPMCA	clinical	Gonzales-Romero *et al*., 2008
	CWD/deer	murine bioassay and sPMCA	clinical	Haley *et al*., 2009
Saliva	scrapie/sheep	sPMCA	preclinical	Maddison *et al*., 2010
	CWD/deer	cervid bioassay	clinical	Mathiason *et al*., 2006
	CWD/deer	cervid bioassay	preclinical	Mathiason *et al*., 2009
	CWD/deer	murine bioassay	clinical	Haley *et al*., 2009
Blood	CWD/deer	cervid bioassay	preclinical	Mathiason *et al*., 2009
	CWD/deer	ELISA and PMCA	preclinical/clinical	Chang *et al*., 2007
	scrapie/sheep	immunocapillary electrophoresis	preclinical/clinical	Jackman *et al*., 2003

	Scrapie/ hamsters	immunoprecipitation and QUiC[(b)]	preclinical/clinical	Orru *et al*., 2011
	scrapie/sheep	sPMCA	clinical	Thorne and Terry (2008)
	scrapie/sheep CWD/deer	PMCA and SOFIA[(c)]	preclinical/clinical	Rubenstein *et al*., 2010
	scrapie/sheep BSE/sheep	rapid ligand-based immunoassay	preclinical/clinical	Terry *et al*., 2009
	scrapie/sheep	Surface-FIDA[(d)]	clinical	Bannach *et al*., 2012
Nasal secretion	TME/hamster	rodent bioassay and QUiC	clinical	Bessen *et al*., 2010
Skin	scrapie/hamster	Western blot	preclinical/clinical	Thomzig *et al*., 2007
	scrapie/sheep	Western blot	clinical	Thomzig *et al*., 2007
	BSE/kudu	rodent bioassay	clinical	Cunningham *et al*., 2004
	BSE/hamster	Western blot	clinical	Thomzig *et al*., 2007
	CWD/elk	rodent bioassay and sPMCA	clinical	Angers *et al*., 2009
Placenta	scrapie/sheep	sheep/goat bioassay	clinical	Pattison *et al*., 1972
	scrapie/sheep	Western blot	preclinical	Tuo *et al*., 2001
	scrapie/sheep	Western blot, IHC	preclinical/clinical	Tuo *et al*., 2002
	scrapie/sheep	ELISA	preclinical	Andréoletti *et al*., 2002
Rectal mucosa	scrapie/sheep	IHC, Western blot, HerdChek CWD EIA[(e)]	preclinical/clinical	Gonzalez-Romero *et al.* 2008a
lymph nodes[(f)]	scrapie/sheep	IHC	preclinical	Andreoletti *et al*., 2000

(a) Protein Misfolding Cyclic Amplification (PMCA).
(b) QUiC (Quake-induced Conversion).
(c) SOFIA (Novel surround optical fibre immunoassay).
(d) Surface-FIDA Fluorescence intensity distribution analysis.
(e) HerdChek CWD EIA: HerdChek CWD (chronic wasting disease) antigen EIA (enzyme immunoassay) test.
(f) Gut-Associated Lymphoid Tissues

Almost all existing tests detect PrP^{Sc} within biological samples either directly or after concentration of the PrP^{Sc} through precipitation (e.g. NaPTA) (Cronier *et al*., 2008, Wadsworth *et al*., 2006, 2001). Alternative approaches for the detection of the disease marker PrP^{Sc} were introduced into prion research studies through the *in vitro* conversion assays. Various *in vitro* conversion assays were developed to understand the mechanism of PrP^{Sc} propagation and a method to improve early non- invasive diagnosis of prion diseases particularly in pre-clinical cases (Table 1.4) (Ryou and Mays, 2008). Each assay has a different methodology and degree of sensitivity; and the so called quaking-induced conversion (QUIC) method and protein misfolding cyclic amplification (PMCA) are the most widely used techniques. In the QUIC technique, the method depends on incubating prion-infected crude brain homogenate with rPrP at 45ºC along with periodic vigorous agitation of the sample. In PMCA, prion-infected crude brain homogenate is incubated with normal, TSE-free crude brain homogenate or rPrP at 37ºC and subjected to repeated pulses of sonication. For both methods, prion amplification is typically carried out over multiple days. These assays have shown great potential for the development of an ultra-sensitive diagnostic test through amplification of prion *in vitro*.

1.8.3. Protein misfolding cyclic amplification (PMCA):

The principal idea of PMCA is to mimic pathological processes and is analogous in principle to the polymerase chain reaction (PCR) used to amplify DNA (but without addition of an enzyme). The first development of this technique by Saborio *et al*., 2001, allowed rapid conversion of part of a large excess of PrP^{C} substrate (a brain extract from the same species) into a protease-resistant, PrP^{Sc}- like form in the presence of minute quantities of PrP^{Sc} template. This conversion occurred as a result of repeat cycles of sonication and incubation at 37ºC. It is hypothesised that with each sonication, the PrP^{Sc} aggregates are disrupted to form multiple smaller units that act as a template capable of initiating further recruitment of PrP^{C} for conversion into PrP^{Sc} (Soto *et al*., 2002) (Figure 1.3).

Table 1.4: Summary of *in vitro* assays for PrP^C conversion and PrP^{Sc} formation (adapted from Ryou and Mays, 2008)

Conversion Method	Conversion Buffer	Incubation	Sonication/ Agitation	PrP^C Source	PrP^{Sc} Source	Percent Converted/ Amplified	Infectivity	Reference
Mixing	PBS with protease inhibitors	37°C ≤ 24 hr		Lysate of N2a cells expressing MHM2 PrP^C	PrP27-30 purified from prion-infected mouse brains	0%		Raeber *et al.*, 1992
Metabolic Radiolabeling	PBS with protease inhibitors	37°C ≤ 24 hr		Lysate of ScN2a cells expressing [35S]- PrP^C	Endogenous PrP^{Sc} of ScN2a cells	0%		Raeber *et al.*, 1992
Microsomal Membranes	20 mM Tris buffer, pH 7.5	25°C 1 hr		[35S]-PrP^C synthesized by cell-free translation systems	Microsomal membranes from scrapie-infected hamster brain cells.	0%		Raeber *et al.*, 1992
Cell-Free Conversion	0.75 M GdnHCl, 130 mM NaCl, 10 mM Tris-HCl, pH 7.0	20°C 22 hr		[35S]-PrP^C expressed in mouse fibroblast cells	Brain-derived PrP^{Sc} treated with 2-3 M GdnHCl for 5 h at 37°C	10 - 20% of PrP^C converted	No	Kocisko *et al.*, 1994
Cell-Lysate Conversion	50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5% SDS	37°C 48 hr		Lysate of CHO cells expressing MHM2 PrP	Brain-derived mouse PrP^{Sc}	Successful, not quantified		Saborio *et al.*, 1999
PMCA	PBS with 0.05% Triton X-100, 0.05% SDS, protease inhibitors	37°C 16-48 hr	40 sec sonication	Normal, uninfected crude brain homogenate	Prion-infected crude brain homogenate	~ 20 -100 fold increase of PrP^{Sc}	Yes	Saborio *et al.*, 2001, Castilla *et al.*, 2005, 2006
PMCA under non-denaturing conditions	PBS with 1% Triton-X 100, 0.5 mM EDTA	37°C 16-48 hr	Continuous agitation, 800 rpm	Purified brain -derived PrP^C	PrP27-30	~10-fold increase of PrP^{Sc}	Yes	Lucassen *et al.*, 2003, Deleault *et al.*, 2005
rPrP-PMCA	PBS with 0.05-0.1% SDS, 0.05-0.1% Triton X-100	37°C 24 hr	40 sec sonication	rPrP expressed in *E.coli*	Purified PrP^{Sc} or crude homogenate of prion-infected brains	~10% of rPrP converted; fold increase of PrP^{Sc} not quantified		Geoghegan *et al.*, 2007
QUIC	PBS with 0.05% SDS, 0.05% Triton X-100	45°C 46 hr	10 sec agitation, every 2 min.	rPrP expressed in *E.coli*	Prion-infected crude brain homogenate	Variable, sensitive to environmental conditions		Atarashi *et al.*, 2008

Further technique development was achieved by high numbers of rounds of sonication through a programmable sonicator and periodic dilution of amplified PrP^{Sc} with fresh substrate materials after each round (a technique termed serial PMCA or sPMCA). This modified technique maximised the rate of PrP^{Sc} amplification in an autocatalytic manner and allowed the detection of minute amounts of PrP^{Sc}: as little as 20 fg/ml, or as few as 4 x 10^5 equivalent molecules of PrP^{Sc} per millilitre (Castilla *et al*., 2005a). These modifications enabled high level amplification and sensitive detection of PrP^{Sc} in different biological materials from scrapie infected rodents in the clinical and preclinical stages of disease, including detection of PrP^{Sc} in brain tissue, blood, urine and faeces (Gonzalez-Romero *et al*., 2008, Maluquer de Motes *et al*., 2008, Murayama *et al*., 2007, Saa *et al*., 2006, Castilla *et al*., 2005b, Soto *et al*., 2005).

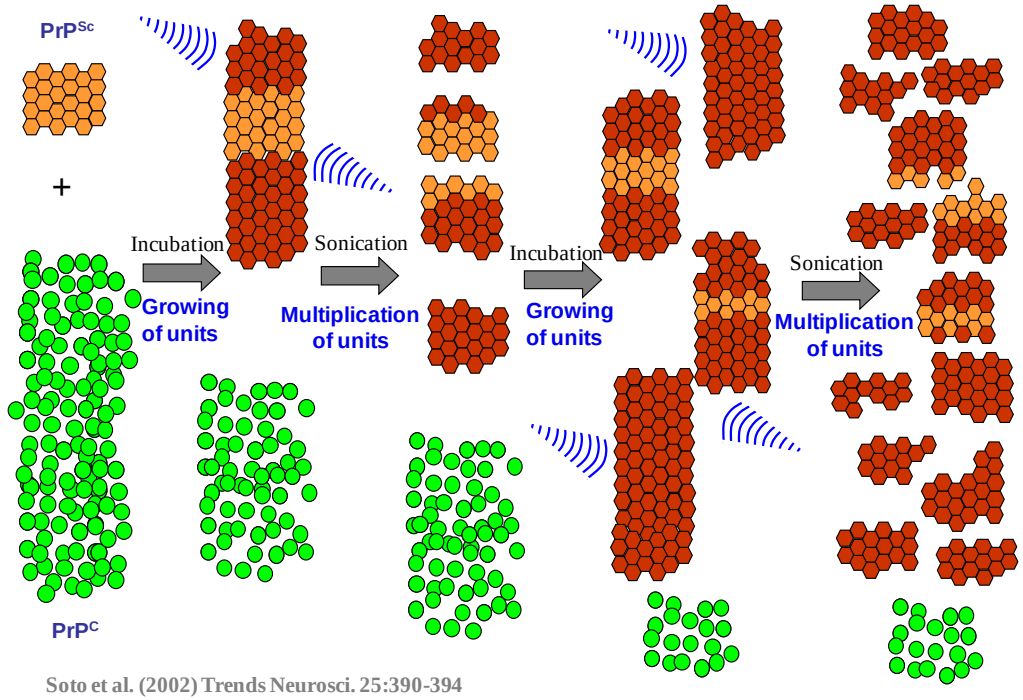


Figure 1.3: Diagram for the mechanism of amplifying PrP^{Sc} by PMCA through subjecting a sample containing minute quantities of PrP^{Sc} and a large excess of PrP^{C} to cycles of incubation and sonication (adapted from *Soto et al., 2002*).

Moreover, in rodent scrapie amplification by PMCA, the addition of polyA or host-encoded RNA molecules has increased the efficiency of amplification. This phenomenon has raised the attention to the cellular factors other than PrP^{C}, particularly specific RNA molecules that might be needed for efficient PrP^{Sc} production (Deleault *et al*., 2007 & 2005). However, the addition of polyA to enhance amplification of sheep scrapie resulted in spontaneous (de novo) synthesis of PK-resistant forms of PrP from partially purified PrP^{C}, adding a limitation to the wide application of PolyA (Thorne and Terry, 2008).

The technique also demonstrated successful amplification of PrP^{Sc} from a range of mammalian species including scrapie in sheep (Thorne and Terry, 2008), CWD in deer (Angers *et al*., 2009, Haley *et al*., 2009, Kurt *et al*., 2007), BSE in cattle (Soto *et al*., 2005) and vCJD in humans (Jones *et al*., 2009 & 2007). Recently, it has been applied to detect PrP^{Sc} in different animals' tissues, secretion and excretion including: blood (Thorne and Terry, 2008, Chang *et al*., 2007), milk (Maddison *et al*., 2009), saliva from clinically normal scrapie-exposed sheep (Gough *et al*., 2012, Maddison *et al*., 2010) and faeces (Terry *et al*., 2011). Furthermore, it has allowed the detection of PrP^{Sc} within environmental samples (Maddison *et al*., 2010, Gough and Maddison 2010). Moreover, further modification to sPMCA have successfully demonstrated the use of bacterially-expressed rPrP as a substrate (Orrú *et al*., 2011, Wang *et al*., 2011, Kim *et al*., 2010, Wang *et al*., 2010, Wilham *et al*., 2010, Orru *et al*., 2009, Atarashi *et al*., 2008, Atarashi *et al*., 2007). Within these studies, experimental animal infections together with the PMCA technique have enhanced the understanding of PrP^{Sc} dissemination as well as providing a technique for the potential ante-mortem diagnosis of preclinical prion disease.

1.8.4. Quaking-induced conversion (QUIC):

Similarly to PMCA, the standard QUIC (S-QUIC) assay depends on propagation of PrP^{Sc} using a $rPrP^{sen}$ as the substrate and replaces sonication with automated intermittent tube shaking to induce the conversion of $rPrP^{sen}$ to $rPrP^{res}$ (Atarashi *et al*., 2008). Initially, the assay was tested to detect PrP^{Sc} in brain homogenate and cerebrospinal fluid (CSF) of scrapie-affected hamsters. It showed similar sensitivity to rPrP-PMCA to detect sub-femtogram levels of PrP^{res}, with clear discrimination between uninfected and scrapie-affected hamster CSF samples, in addition to the

advantage of simplicity and more speed than conventional PMCA (Atarashi *et al*., 2008). Further adaptation of the QUIC reaction was carried out for the detection of prions in CSF of human CJD and sheep scrapie (Orru *et al*., 2009), and nasal lavages from HY (hyperaethesia strain; a short-incubation-period strain) TME-infected hamsters (Bessen *et al*., 2010). Further development of the S-QUIC assay for estimating the relative amount of prions resulted in the advent of a real-time quaking induced conversion assay (RT-QuIC) (Wilham *et al*., 2010). RT-QuIC has the advantage of a lack of the use of chaotropic salts like SDS (virtually detergent-free; <0.002% SDS) that is responsible of stabilizing $rPrP^{res}$ polymers which might affect efficiency of conversion, and GdnHCL (which reduces the potential for false positive reactions) (Atarashi *et al*., 2011 a & b, Wilham *et al*., 2010). It also depends on ThT (thioflavin T) based fluorescence detection of prion seeded $rPrP^{sen}$ amyloid fibrils. The assay has been used to detect prions of sheep, deer and hamster with a high sensitivity similar to animal bioassays (Wilham *et al*., 2010). Furthermore, RT-QuIC showed promising enhanced diagnostic capacity for the detection of PrP^{Sc} in CSF from patients with CJD (McGuire *et al*., 2012, Atarashi *et al*., 2011), and in human and sheep plasma when coupled with a immunoprecipitation step (Orru *et al*., 2011). However, although the QuIC assay is of high sensitivity and specificity, further investigations of the variation in sensitivity in detection of different forms of CJD in humans, particularly vCJD, are required (Peden *et al*., 2012, Orru *et al*., 2011)

In summary, the exhibited diverse heterogeneous prion strains in ruminants, particularly scrapie in sheep, has initiated a substantial amount of research aimed at producing diagnostic tests with higher sensitivity and specificity, after concentrating or amplifying the PrP^{Sc}. Within these areas, Western blot protocols, ELISA tests, and IHC protocols were initially validated by examination of samples for PrP^{Sc} detection through its protease resistance and identification by use of PrP-antibodies. Furthermore, the reliability of these tests for detection of prion in non neural tissues, clinical samples at an earlier stage in the incubation period and environmental samples have opened more avenues for a better understanding and accurate diagnosis of prion diseases in sheep. The limited knowledge of diverse scrapie strains and the need for early diagnostics methods in the field made scrapie in sheep an interesting host to study.

1.9. Aims of the study:

Based on the latest published research reports regarding diagnosis of TSEs in animals, this study will research the characterisation of prion diseases in sheep through:

1. **The development of an ELISA for the detection of PrP^{Sc}:**

Digesting PrP^{Sc} by the protease TH resulted in full length PrP^{Sc} and digested PrP^{C} (Owen et al., 2007). In order to detect full length PrP^{Sc} by ELISA, I looked at digesting scrapie infected brain homogenate samples with the protease TH. To separate the two isoforms; full length PrP^{res} and fragmented PrP^{C}, PrP^{res} would be precipitated with NaPTA, isolated from PrP^{C} fragments and then detected within the sandwich ELISA using capture antibody directed towards an epitope in the N-terminal part of PrP^{Sc} and a detection antibody directed against the PrP^{Sc} core. The success of developing this ELISA to detect PrP^{res} in brain homogenate will further extend to detect amplified products of seeded PMCA reaction.

2. **Defining molecular differences of experimental and natural strains/isolates of ovine prion diseases through differential protease resistance and Western blotting:**

Using PK and TH proteases for digesting PrP^{Sc} and analysing the products by Western blotting using a range of antibodies targeted to the N-terminal and core regions of PrP have enabled to differentiate between classical scrapie and ovine BSE (Owen *et al*., 2007, Thuring *et al*., 2004). But, the molecular profile of PrP^{res} in Western blots showed that PK PrP^{res} from sheep inoculated with CH1641 scrapie gave a molecular profile similar to that of sheep inoculated with BSE (Hope *et al*., 1999, Stack *et al*., 2002, Lezmi *et al*., 2004). Since each protease has different proteolytic activity against PrP^{Sc}, resulting in truncated PrP^{res} fragments with PK and full length PrP^{res} with TH, I hypothesised that digesting CH1641 and ovine BSE samples with TH will generate unique PrP^{res} molecular profiles for each strain. This approach will help in developing a strain typing assay capable of distinguishing between CH1641 from ovine BSE and ovine scrapie.

3. **Characterising experimental and natural strains/isolates of ovine prion diseases by amplification within PMCA and using natural PrP^C substrates:**

PMCA has achieved a considerable success in the *in vitro* replication of rodent adapted scrapie and detecting low level of prion in biological materials. Previous research reports have showed that different factors have been implicated in the success of PrP^{Sc} replication. This includes: prion strains and PrP^C substrate genotype (Thorne and Terry, 2008) and cross species compatibility between the PrP^{Sc} seed and PrP^C substrate (Soto *et al*., 2005). In ruminant TSE diseases, it has not been used in a large scale to assure the efficient replication of varieties of TSE strains in ruminants. Therefore, different strains of ruminant prions will be used to seed PMCA reactions to elucidate the potential amplification of these strains and detection of low levels of PrP^{Sc}. I will also investigate:

1) The effect of prion strain, host species and *Prnp* genotype on prion replication.
2) The differentiation between ovine BSE and CH1641 through amplification within natural PrP^C substrates of different *Prnp* genotypes.

4. **The potential of amplification of experimental and natural strains/isolates of ovine prion diseases by PMCA within rPrP substrates:**

Due to the difficulty of obtaining a continuous supply of crude brain homogenate as a substrate, there are different trials that showed successful use of rPrP expressed in bacteria as a substrate in cell free conversion assays (Atarashi *et al*., 2007, Atarashi *et al*., 2008, Kim *et al*., 2010, Orrú *et al*., 2011, Orru *et al*., 2009, Peden *et al*., 2012, Wang *et al*., 2010, Wang *et al*., 2011, Wilham *et al*., 2010). Therefore, I will test the use of bacterially expressed rPrP as a substrate through expression and purification of rSha PrP and ovine VRQ rPrP. These rPrP proteins will be used as substrates in PrP^{Sc} seeded sPMCA reactions with a range of sheep prion seeds to generate PK $rPrP^{res}$ fragments.

Chapter 2: MATERIALS AND METHODS

2.1. Source of CNS materials:

TSE-infected ruminants were sourced from the Veterinary Laboratories Agency (VLA) transmissible spongiform encephalopathy (TSE) archive or collected prospectively from confirmed TSE-infected ruminants submitted to the VLA for diagnosis. All samples were confirmed negative or positive by routine diagnosis using Bio-Rad TeSeE ELISA, immunohistochemistry and VLA hybrid Western blot (Stack *et al.*, 2002). Experimentally infected animals were euthanized following onset of symptoms, sampled within 60 minutes of euthanasia and samples snap frozen at -80°C. All animal procedures were performed under Home Office (UK) and local ethical review committee approval and compliance with the Animal (Scientific Procedures) Act 1986.

2.2. Brain tissue homogenate preparation:

Brain tissues from healthy or prion-infected sheep were homogenised to 10% (w/v) in lysis buffer (0.5% Nonidet P-40 (v/v) (Igepal CA-630, Cat No. I8896, Sigma) and 0.5% (w/v) sodium deoxycholate (Cat No. 5670, Sigma) in PBS, pH 7.4) in a microbiological safety cabinet. The resulting suspension was passed through needles of decreasing diameters (19-, 21-, and then 23-gauge needles), and the samples were centrifuged for 5 minutes at 270 g to remove cellular debris. The supernatants were removed carefully and stored in 1 ml aliquots at -20˚C.

2.3. PMCA brain substrate preparation:

Healthy sheep brains were prepared from a New Zealand-derived scrapie-free flock. Whole brain was cleared of attached membranes and all superficial blood vessels and then washed with cold PBS. The brain was placed into ice cold conversion buffer (50 mM NaCl, 4 mM Ethylene diamine tetraacetic acid (EDTA), pH 8.0, 1.0% (v/v) Triton X-100 and miniprotease inhibitor, Roche in PBS pH 7.4) and immediately homogenized at 10% (w/v) using a liquidiser. For further homogenisation, the liquidised brain was placed into a bead beater in batches and each batch was homogenised for 30 seconds. After ensuring full tissue homogenisation, any cellular debris was removed by a low speed centrifugation at 600 *g* for 10 minutes at 4°C. The supernatant was removed into a clean 1 Litre bottle placed into an ice bath, and

mixed thoroughly. Prepared homogenates were placed into 2 ml eppendorf tubes and stored immediately at -80ºC.

2.4. Automated serial PMCA (sPMCA):

Different volumes/dilution series of 10% (w/v) infected brain tissues from sheep with distinct PrP genotypes were spiked into brain homogenate substrate (10% (w/v) brain homogenate from sheep with different PrP genotypes or bovine samples in PMCA conversion buffer (50 mM NaCl, 4 mM EDTA, pH 8.0, 1.0% (v/v) Triton X-100 and miniprotease inhibitor, Roche in PBS pH 7.4) or rPrP substrate [0.1 or 0.2 mg/ml rPrP in conversion buffer (PBS (pH 7.4) with 0.05% (w/v) Sodium dodecyl sulfate (SDS) and 0.05% (v/v) Triton X-100 and sucrose up to 6% (w/v)]. Each spiked sample together with non-spiked control substrate samples were loaded into 200 µl PCR tubes (Dutscher Scientific, West Thurrock, UK). All samples were placed in an ultrasonicating water bath (model 3000; Misonix) at 37ºC and sonicated for 40s at 200W. Sonications were repeated once every 30 minutes for 24 hours (one PMCA round). After each round the sonicated samples were gently pulsed in a microfuge. Samples were divided into two parts; one part was frozen at -20˚C and the other part was diluted 1: 3 with 10% (w/v) brain homogenate substrate or rPrP substrate (0.1 or 0.2 mg/ml) to a final volume of the original spiked substrate samples. Samples were then subjected to further rounds of PMCA. The number of PMCA rounds was typically 3 to 10. Further samples were prepared in exactly the same way as for the PMCA samples, but were to act as controls to show the efficiency of PMCA amplification. These samples were not subjected to amplification. All amplified samples were stored at -20ºC. All samples were digested with PK, and PrP^{res} was detected by immunological methods; Western blot or ELISA.

2.5. Optimising proteinase-K (PK) and thermolysin (TH) protease for the complete clearance of ovine PrP^{C} or rPrP:

Aliquots of brain homogenates (20 µl of 10% w/v) from healthy sheep or rPrP (10 µl of 0.1 mg/ml) were treated with dilution series of PK and/or TH separately to assess for the complete clearance of PrP^{C}. PK digestions were carried out at final concentrations between 1 and 100 µg/ml protease for 1 h at 37ºC and digestion

stopped by boiling the samples at 100ºC for 5 minutes. When assessing the digestion of rPrP, digestions were carried out +/- 0.01% (w/v) SDS. TH (Sigma, Poole, United Kingdom) digestions were carried out at final concentrations between 10 and 150 µg/ml protease for 1 h at 70°C, and digestion was stopped by adding EDTA to a final concentration of 10 mM.

Each digested sample was diluted with two volumes of NuPAGE 2x LDS sample buffer containing 5% (v/v) β-mercaptoethanol (Invitrogen, Paisley, United Kingdom), and the samples heated to 100°C for 10 minutes. Typically, 3.3 µl of each digested 10% (w/v) brain homogenate sample or 5 µl of digested rPrP sample were analyzed by Western blotting.

2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting:

SDS-PAGE was carried out through a running gel containing 12% (w/v) total acrylamide using NuPAGE precast Bis-Tris gels (Invitrogen, Paisley, United Kingdom). Separated proteins were transferred to a polyvinylidene difluoride membrane (Roche, Lewes, United Kingdom) by using a NuPAGE blot module (Invitrogen, Paisley, United Kingdom) at 30 V for 1 h. All membranes were blocked with 15 ml of 5 % (w/v) powdered milk baby formula (SMA) (in Tris-base buffer (8 g NaCl, 0.2 g KCl, 3 g Tris base, dissolved in distilled water up to 1 Litre and pH was adjusted to 8.0) containing 0.05% (v/v) Tween 20 (TBST) overnight at 4˚C. Each blot was probed with a primary monoclonal antibody (mAb) diluted in 0.5 % (w/v) SMA-TBST , that is specific for epitope in the ovine PrP. Membranes were incubated with one of the primary mAbs (Table 2.1 and Figure 2.1) for two hours with shaking. After 3 times washing with 0.5 % (w/v) SMA-TBST, membranes were incubated with goat anti-mouse horseradish peroxidase (HRP) conjugate (DakoCytomation, Glostrup, Denmark) diluted in 0.5 % (w/v) SMA-TBST, for 75 minutes with gentle shaking. After three washes with 0.5 % (w/v) SMA-TBST, membrane was treated with 5 ml EZ- chemiluminescence for HRP substrate (Gene flow, Staffordshire, UK) for 5 minutes. Bound anti-PrP antibodies were visualised after exposure of the membranes on Fuji X- ray films (Fisher Scientific, Leicestershire, UK) or using an ICCD225 camera system from Photek Ltd., (Photek Ltd., East Sussex, UK).

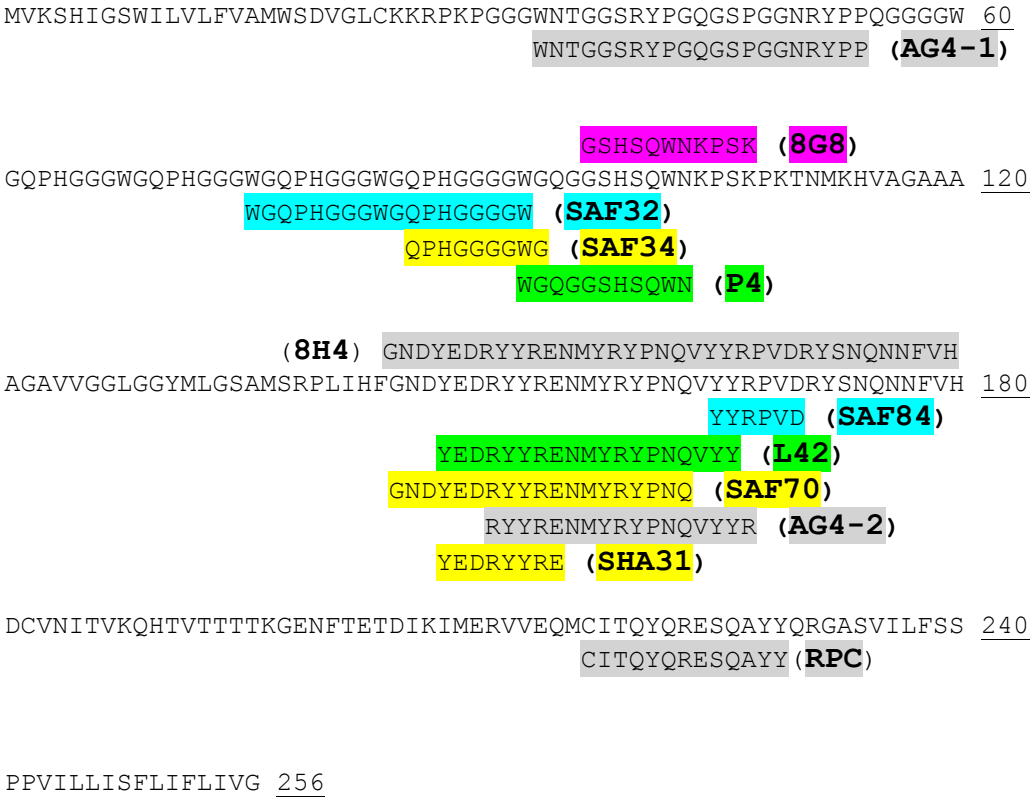


Figure 2.1: Epitope recognition sequences map for Anti-prion antibodies: AG4 (AG4-1: N- terminal epitope and AG4-2: C- terminal epitope), SAF32, SAF34, P4, 8G8, 8H4, SAF84, L42, SAF70, SHA31 and Rabbit polyclonal antibody (RPC) in relation to the primary amino acid sequence of ovine prion protein (EMBL accession number P23907).

Table 2.1: Characteristics of monoclonal antibodies (mAbs) used for Western blotting:

Antibody	Linear epitope on PrP	Immunogen	Species tested and recognised***	Antibody concentration (working dilution)	Reference (s)
SAF32	59 - 89	Ha SAF	Ov, Bov, Mo, Ha and Hu	1: 40,000	Feraudet *et al*., 2005
SAF34	59 - 89	Ha SAF	Ov, Bov, Mo, Ha and Hu	1: 40,000	Feraudet *et al*., 2005
P4	93 - 99	Ov PrP Peptide	Ov and Bov	1: 5,000	Harmeyer *et al*., 1998, Thuring *et al*., 2004
AG4	31 - 51 along with 147 - 163	Ov full length rPrP	Mo and Hu	1: 2,000	TSE Resource Center, UK. Maddison *et al*., 2007
SHA31	145 - 152	Ha SAF	Mo, Ha and Ov	1: 40,000	Feraudet *et al*., 2005
SAF70	145 - 162	Ha SAF	Mo, Ha and Ov	1: 40,000	Feraudet *et al*., 2005
SAF84	126 - 164* or 166 - 172**	Ha SAF	Mo, Ha and Ov	1: 40,000	Feraudet *et al*., 2005, Jacobs *et al*., 2011
8G8	97 - 102 or 100 - 107	rec Hu PrP	Ov, Bov, Mo, Ha and Hu	1: 10,000	Feraudet *et al*., 2005 and Gretzschel *et al*., 2006
8H4	145 - 180		Hu	1: 10,000	
L42	148 - 153	Ov PrP peptide	Ov, Bov, Mi and Hu	1: 1,000	Harmeyer *et al*., 1998 , Gretzschel *et al*., 2006
Rabbit polyclonal antibody (RPC)	217 - 229	Bov PrP	Ov, Bov	1 µg/ml	Fisher Scientific

* Recognise solid-phase immobilized peptide 126-164, but failed to bind peptide 142- 160 of hamster PrP (Feraudet *et al*., 2005).
** Assessed by solid phase peptide mapping (Pepscan analysis) and binding is highly dependent on 171Q containing allelotypes (Jacobs *et al*., 2011).
*** Ov = ovine, Bov = bovine, Mo = mouse, Ha = hamster, Hu = human, Mi = mink and SAF = scrapie associated fibrils.

2.7. Expression and purification of VRQ full length rPrP:

2.7.1. Bacterial culture:

A glycerol stock of Novablue DE3 containing plasmid pET22b-VRQ1-3 was provised by ADAS biotechnology group. The VRQ1-3 clone (expressing PrP 23-231) was cloned into the Nde1/BamH1 sites in pET22b. The expression is driven by a T7 polymerase promoter on the pET22b, and IPTG induces expression of T7 polymerase, which then drives high level expression of the gene under T7 control. This expression vector clone contains the ovine *Prnp* gene coding for the protein with the VRQ polymorphisms (expressing amino acids 23-231 and lacking the N- and C-terminal signal sequences). The expression vector clone was streaked out onto a Luria-Bertani- agar (LB) plate containing 50ug/ml ampicillin (Melford, Suffolk, UK), the plate was incubated at 37ºC overnight. On the following day, the plate was stored at 4ºC until use.

2.7.2. Induction of protein expression:

A single colony of *E. coli* was picked and inoculated into 5 ml 2x YT-A-G medium [2xYT medium (16 g Bacto tryptone (Oxoid), 10 g Bacto yeast extract (Oxoid), 5 g NaCl, up to 1 litre distilled water, pH 7.0, then autoclaved) containing ampicillin (Melford, Suffolk, UK) (up to 100 µg/ml; 5 µl 100 mg/ml) and glucose (up to 3% glucose). Four Falcon tubes were prepared, incubated at 37ºC overnight with shaking in the incubator at 200 rpm. In addition, 10 ml of the 2x YT-A-G medium without bacteria was kept as an absorbance reference for cell growth monitoring.

1 ml of the overnight culture was inoculated into each of 4 x 250 ml 2YT-A-G (250 ml 2 YT + 25 ml glucose + 125 µl ampicillin) in 4 flasks (1L unbaffled flask).

In addition, 0.3 ml of the overnight culture was inoculated into each of 4 x 250 ml 2 YT-A-G in 4 flasks (1 L non baffled flasks). All flasks were incubated at 37ºC with shaking in the incubator for 2 to 4 hours or until absorbance at 595nm reaches ~ 0.4. Absorbance reference was measured against 2x YT-A-G medium without bacterial growth.

After an absorbance of ~0.4 was reached, the cultures were transferred into sterilized bottles and centrifuged at 1810 g for 30 minutes to pellet cells.

The cell pellets were re-suspended in 2YT-A-IPTG [250 ml 2YT + 125 µl ampicillin + IPTG (Isopropyl-B-D-thiogalactopyranoside) (Melford, Suffolk, UK) for a final

concentration of 1.0 mM]. Each bottle received 50 ml 2YT-A-IPTG media to re-suspend the cell pellets and transferred to a flask containing 200 ml of 2YT-A-IPTG media. The inductions were incubated at 37ºC overnight with shaking at 250 rpm in the incubator.

2.7.3. Cell lysis and isolation of inclusion bodies:

2.7.3.1. Cell harvesting:

The media were transferred into sterilized 500 ml centrifuge bottles and centrifuged at 1810 g for 30 minutes to pellet cells.

2.7.3.2. Enzymatic digestion of the cell membrane:

After centrifugation, the supernatant was discarded. The culture pellets were thoroughly re-suspended in 1 ml lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 0.1% Nonidet P-40, (10 mg/ml) 1 Lysozyme (Sigma), Roche Complete Mini tab, (EDTA-Free; Sigma)) per 50 ml culture volume (i.e. 20 ml per 1 litre). The pellets were re-suspended with the lysis buffer at room temperature by pipetting up and down with a 25 ml pipette. Cell lysates were poured into a beaker and frozen at -20˚C for 20 minutes and thawed at least one time.

DNase I (Deoxyribonuclease) (Boehringer Mannheim, Germany) was added up to 20 µg/ml (i.e. 800 µg/ 40 ml of the cell lysate) with $MgCl_2$ to 10 mM and the mixture was stirred at room temperature until the liquid became viscous. The lysates were divided between 50 ml centrifuge tubes (8 tubes) and the viscous liquid was centrifuged to pellet inclusion bodies at 12000 g for 20 minutes.

After centrifugation, the supernatant was discarded. Each pellet was washed by thorough re-suspension (vortex) in 25 ml washing buffer (0.1% Nonidet P-40, 10 mM Tris pH 8 up to 2 litres and incubated on ice for 20 minutes before use) per pellet, and re-centrifuged again at 12000 g for 20 minutes.

The washing process was repeated up to 5 times until pellets became white. The supernatants were removed and the pellets were well drained, resulting in pellets containin rPrP precipitated in the form of inclusion bodies which were stored overnight at 4˚C.

2.7.3.3. Solubilising the PrP-containing inclusion body pellets:

Pellets were solubilised in 20 ml of urea buffer (9 M urea, 20 mM Tris HCl pH 8 and 100 mM DTT). Using a Pasteur pipette, the urea solubilising buffer was gently

pipetted across the pellet surface until covering the pellet volume (5 ml urea solubilising buffer per pellet) and this step was repeated until all was re-suspended, avoiding bubbles. To complete the solubilisation step the samples were mixed overnight by rotation at 4˚C.

5 ml of the solubilised pellet solution was centrifuged to pellet insoluble debris at 12000 g for one hour. The supernatant was removed and added to 15 ml 8M urea solubilising buffer in preparation for purification through a Fast Protein Liquid Chromatography (FPLC) system (AKTA prime, GE Healthcare).

2.7.4. Purification of rPrP protein:

The purification method is based on the affinity of the conserved octapeptide repeats in the N-terminus of the prion protein for transition-metal cations (Viles *et al*., 1999). This metal affinity was exploited to purify full length rPrP through binding of a tandem repeat sequence (PHGGGWGQ) to copper ions by immobilised metal affinity chromatography (IMAC). A 5 ml chelating sepharose column was used (IMAC HP, GE Health care, Buckinghamshire, UK) to immobilise copper ions. The column was assembled on the FPLC machine, and was loaded with copper by passing through a solution of copper sulphate (50 mM $CuSO_4$), the column was washed and then primed with denaturing buffer (8 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, pH 7.5). The sample (20 ml) was loaded as a denatured protein in the urea solution at flow rate 0.2 ml per minute. After loading the sample, the protein was refolded using a gradient wash step using a urea gradient from 8 M to 0 M over 2.5 hours [The gradient washing steps were done initially at a rate of 0.5 ml per minutes denaturing buffer passing through the column for 10 minutes, followed by 1 ml per minute from denaturing buffer to non-denaturing buffer (i.e. refolding step) for 140 minutes]. Refolded rPrP was eluted in an imidazole elution gradient from 0 - 0.5 M imidazole elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.5 M imidazole, pH 7.5), at a flow rate of 0.2 ml per minute for 100 minutes and fractions (1 ml each) were collected using an automated fraction collector. Samples were stored at 4ºC.

Protein fractions corresponding to a protein peak elution on the A280 chart recorder were taken and pooled. The concentration of this purified protein was measured by Bradford assay (Cat. No. B6916, Sigma) against a BSA standard. The purity of the final product was determined by SDS - PAGE. i.e. 30 µl of NuPAGE 2x LDS sample

buffer containing 5% (v/v) β-mercaptoethanol (Invitrogen, Paisley, UK) was added to 10 µl of selected samples. Samples were heated to 100°C for 5 minutes. All samples were stored at 4°C for further analysis. Typically, 5 µl of each sample was loaded into SDS-PAGE and electrophoresis was carried out through a running gel containing 12% (w/v) total acrylamide using NuPAGE precast Bis-Tris gels (Invitrogen, Paisley, UK) at 200 V for 1 hour. Separated proteins were stained with Coomassie blue stain. Samples containing pure rPrP were mixed with sucrose (up to 60%) and then aliquoted and stored at -80ºC.

This production and purification of VRQ rPrP procedure was carried out during this study. Other preparations of VRQ rPrP as well as rPrP of distinct ovine genotypes and hamster rPrP were carried out by other members of the research group and the pure proteins used in this study.

2.8. Development of a quantitative ELISA to measure PrP:

The following represents the typical ELISA protocol developed in the current study. ELISA plates (Nunc, Fisher Scientific) were coated with 100 µl/well of anti-PrP antibody diluted in PBS as a capture antibody. Plates were incubated at 20˚C overnight. Plates were blocked with 400 µl of blocking agent diluted in PBS containing 0.05% (v/v) Tween 20 (Cat No. P7949- Sigma) (PBST) for 1 h at room temperature. Test samples containing PrP^{Sc} were treated with the protease TH and then precipitated with sodium phosphotungstic acid (NaPTA) (as described by Cronier *et al*. 2008), before re-suspension in the original buffer volume. All samples were denatured and spun at 11393 g for 5 minutes to precipitate any non-soluble aggregates. For all samples, 60 µl of each denatured sample supernatant was further diluted to 600 µl in PBST (dilution buffer) and 200 µl of this (typically containing 5 µl of biological material) was added to ELISA wells in triplicate and incubated at room temperature for 1 h. Bound PrP was detected with 100 µl of antibody SHA31 (recognising ovine PrP residues 145 - 152) diluted in blocking buffer and incubated for one hour at room temperature. Bound SHA31 was detected with rabbit anti-mouse IgG1-alkaline phosphatase conjugate (anti IgG1 isotope specific antibody, Invitrogen, Carlsbad, CA92008, USA) diluted in blocking buffer (1: 2,000). All wash steps consisted of five washes with PBST. Bound conjugate was visualised with p-

Nitrophenyl phosphate (pNPP) substrate (Cat. no. N-2770, Sigma) and optical density (OD) was measured at an emission of 405 nm on a microplate reader.

This general ELISA procedure was conducted with each step optimised to develop the final ELISA format. The steps which were empirically optimised included the following:

2.8.1. Antibody concentrations, specificity and compatibility:

Typically 100 µl of each coating antibody that recognize ovine PrP were diluted into PBS (pH 7.4) and were assessed at different concentrations ranging from 1: 2,000 to 1: 12,000. The antibodies used were SAF15 (recognising PrP residues 79 - 92, isotype IgG3), SAF32 (recognising PrP residues 79 - 92, isotype IgG2b), SAF70 (recognising PrP residues 142 - 160, isotype IgG2b), SAF84 (recognizing solid-phase immobilized peptide 126 - 164, isotype IgG2b), SAF33 (recognising PrP residues 79 - 92, isotype IgG2b) and SAF34 (recognising PrP residues 79 - 92, isotype IgG2a) (all kindly provided by J. Grassi, CEA Saclay, Gif/Yvette, France) and detection antibody SHA31 (isotype IgG1).

2.8.2. Blocking agent:

With optimised antibody concentrations (see result sections 3.2.1) the blocker of choice was determined; blocking agents were powdered milk baby formula (SMA nutrition, UK), Bovine Serum Albumin (Cat No. A-2153, Sigma) and Chicken Egg Ovalbumin (Cat No. A-5253, Sigma), assessed at different concentrations in different dilution buffers.

2.8.3. Denaturation conditions:

With optimised antibodies and blocking agent concentrations (see result section, 3.2.2.), all healthy brain samples were denatured in different denaturation conditions; these were denaturation through boiling for 5 minutes, denaturation with SDS (Cat No. S1030, Melford, Ipswich, UK) up to a final concentration of 4% (w/v) and incubation at 100°C for 10 minutes, and denaturation with Guanidine hydrochloride (Gdn-Hcl) (Cat No. 4505, Sigma) up to 4% (w/v) and incubation at 80°C for 5 minutes.

2.8.4. Precipitation of PrP^{Sc}:

Digested diseased brain homogenates were precipitated by adding N-lauryl-sarcosyl to 0.1% or 4% and incubation for 30 minutes at 37ºC, and then incubation with NaPTA for 30 minutes at 37ºC. This assessed the recovery and precipitation of aggregated PrP^{Sc}.

2.8.5. Detection of a dilution series of scrapie-affected brain homogenate (standard curve):

This was performed using increasing amounts of diseased brain homogenates diluted into either 10% (w/v) brain homogenate from a TSE-free animal or PBS. Following NaPTA precipitation, all samples were treated with the protease TH (Cat No. P1512, Sigma) at a final concentration of 100 µg/ml for one hour at 70°C, centrifuged at 43 g for 45 seconds, and then the digestion was stopped by the addition of EDTA (to 10 mM).

2.8.6. Detection of ovine VRQ rPrP:

An aliquot of ovine rPrP (VRQ) (0.8 mg/ml) was diluted into PBS to obtain different dilution series ranging from 40 ng to 0 ng per well. Then, all serially diluted samples were denatured and the optimised ELISA procedures applied.

2.8.7. Comparison of PrP detection limits using Western blot and the developed ELISA protocol:

Based on previous optimisation steps, a further experiment was conducted to compare the sensitivity and quantitative detection limit between the developed ELISA assay and a high sensitivity Western blot technique.

Two dilution series of PrP^{Sc} using 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2 µl, and 3 µl volumes of 10% (w/v) scrapie positive brain homogenates were spiked into healthy brain homogenates to a total volume of 5 µl of brain homogenates per well. One dilution series was digested with TH and a second dilution series was digested with PK (50 ug/ml) at 37ºC for one hour. All TH digested samples were processed based on the optimised procedures for precipitating PrP^{Sc} with NaPTA and re-suspension to the original volume of the sample. These were then analysed using the optimised ELISA protocol.

In addition, 30 µl of NuPAGE 2x LDS sample buffer containing 5% (v/v) *B*-mercaptoethanol (Invitrogen, Paisley, UK) was added to 10 µl of all TH and PK digested homogenates, and samples were heated to 100°C for 5 minutes. All samples were stored at 4 °C for further analysis. Typically, 5 µl of digested 10% (w/v) brain homogenate within the dilution series was analysed by Western blot. For PK digested samples, membrane was probed with primary mAb SHA31 (1: 40,000). For TH digested samples, one membrane was probed with primary mAb SHA31 (1: 40,000) and a second membrane was probed with anti N-terminal mAb SAF34 (1: 40,000). Blots were visualized by using an ICCD225 camera (Photek Ltd., East Sussex, UK).

2.8.8. Comparison between high sensitivity Western blotting and the developed ELISA protocol for detecting PMCA- PrP^{Sc}:

To estimate total PrP^{Sc}-PMCA levels, 20 µl of each sample subjected to PMCA reaction was treated with $CaCl_2$ and $ZnSO_4$ (up to a final concentration of 10 mM each), and then digested with TH (100 µg/ml) for one hour at 70ºC, and digestion stopped with EDTA (final concentration 20 mM). A further 20 µl of each sample was treated with SDS (up to final concentration of 0.04%) and digested with PK (50 ug/ml) at 37ºC for one hour. Digestion was stopped with PMSF up to 5 mM.

To remove fragments of PrP^{C}, each digested sample was then precipitated with sodium phosphotungstic acid (NaPTA) as described by Cronier *et al*., 2008, and pellets were re-suspended to their original volume in PBS.

5 µl of each TH digested sample was analysed by the developed ELISA and OD values (absorbences read at 405 nm) were used to record qualititative data; positive or negative for PMCA-PrP^{Sc}. In addition, 5 µl of each PK and TH digested sample were analysed by Western blot. Comparison of the detection result (detected or not detected) of each sample analysed by ELISA to the signal intensity of each sample digested with PK and TH and analysed by Western blot were conducted to compare the sensitivity of detecting PMCA-PrP^{Sc} by each method.

RESULTS

Chapter 3: Development of a Quantitative ELISA to Measure PrP^{Sc}

3.1. Introduction:

The biochemical features of PrP digestion by proteases form the basis for differentiating between PrP^{C} and PrP^{Sc} where PrP^{C} is susceptible to protease digestion and PrP^{Sc} is partially resistant. Treating prion infected samples with PK results in full digestion of PrP^{C}, and N-terminally truncated PrP^{Sc} conformers.

In a sandwich ELISA technique, PrP^{Sc} is captured with an anti-PrP antibody through incubation with immobilised anti-PrP mAb on an ELISA plate. The PrP^{Sc} sample would first be digested with PK followed by denaturation to reveal PrP^{Sc} epitopes. The captured PrP^{Sc} is then recognised by a second anti-PrP antibody and this detection antibody is then labelled with a secondary conjugated antibody. Bound secondary antibody conjugate is then detected using chemiluminescence (Creminon *et al*., 2004) or colorimetric enzyme substrates.

With a conformation dependent immunoassay (CDI) (Safar *et al*., 2005, 2002 & 1998), the technique relies on the differential binding of antibodies to native or denatured PrP^{Sc} rather than resistance or sensitivity to PK. In this assay, a specific antibody will bind to an epitope exposed in native PrP^{C} while the same epitope is buried in native PrP^{Sc}. After denaturation of PrP^{Sc}, this epitope will become exposed and will be recognised by the antibody. These distinct binding patterns lead to quantifiable differences between the ratio of the signals given by denatured PrP^{Sc} and native PrP^{Sc}, compared to those with denatured and native PrP^{C} (Thackray *et al*., 2007, McCutcheon *et al*., 2005, Safar *et al*., 2005, 2002 & 1998). Further modifications of the CDI assay have been developed by incorporating an extraction and concentration step using NaPTA allowing the detection of low concentrations of PrP^{Sc} (Wadsworth *et al*., 2006, 2001).

Within the present study, I have adopted the principle of a sandwich ELISA to detect full length PrP^{Sc} but through treating samples with the protease TH. Owen *et al*., 2007, found that treating PrP^{Sc} infected tissue with TH reveals a full length PrP^{Sc} that is resistant to proteolysis, and digests PrP^{C} into small protein fragments. In addition, extraction of PrP^{Sc} with NaPTA (Cronier *et al*., 2008) removed any PrP^{C} fragments. I was able to develop an ELISA protocol for PrP^{Sc} quantification within prion infected samples.

3.2. Results:

3.2.1. Determining antibody concentrations, specificity and compatibility:

To assess antibody specificity and compatibility, the ELISA plate was incubated with either: (1) no capture or detection antibodies, (2) capture antibody alone, (3) detection antibody alone or (4) both capture and detection antibodies. The pairing of different capture antibodies against the detection antibody SHA31 was assessed as well as the use of different PrP^{Sc} denaturing methods. Dilution series of the detection antibody SHA31 was used to detect 1 µl 10% healthy brain homogenate per well captured by dilution series of capture antibody SAF32 (Graph 3.1). When assessing a panel of IgG mouse monoclonal antibodies for the capture of PrP, SAF15, SAF32 and SAF34, at different concentrations ranging from 1: 2,000 to 1: 12,000, all had successful binding to the N-terminal region of the PrP residue 79 - 92 and captured PrP onto the ELISA plate (Table 3.1).

The optimal dilutions of capture antibody were 1:5,000 - 1:8,000 for SAF15, 1:5,000 – 1:12,000 for SAF34 and 1:8,000 – 1:12,000 for SAF32 (Table 3.2). However, the signals produced were consistently higher for SAF34 compared to the other capture antibodies and this was selected for further method development and used at a dilution of 1:8,000. The bound SHA31 antibody was detected with conjugated Alkaline Phosphatase (AP)-Rabbit Anti-Mouse IgG1 (diluted 1: 2,000 in blocking buffer) which recognised SHA31 but did not cross react with SAF34 or the other capture antibody (data not shown).

Graph 3.1: Determining detection antibody optimum dilutions within a sandwich ELISA format: Dilutions series from 1: 1,000 to 1: 8,000 for detection antibody SHA31 against capture antibody SAF32 1:2,000. Signals generated with 1 µl 10% (w/v) healthy brain homogenate in PBS per well were compared to background signals (no brain homogenate). All samples were added to ELISA wells in triplicate. Further controls (0) included omitting capture and detection antibodies. Through comparing absorbance densities at 405 nm, there was little difference between different detection antibody concentrations, so the optimised dilution for SHA31 detection was determined to be 1: 8,000 (This experiment was done once only).

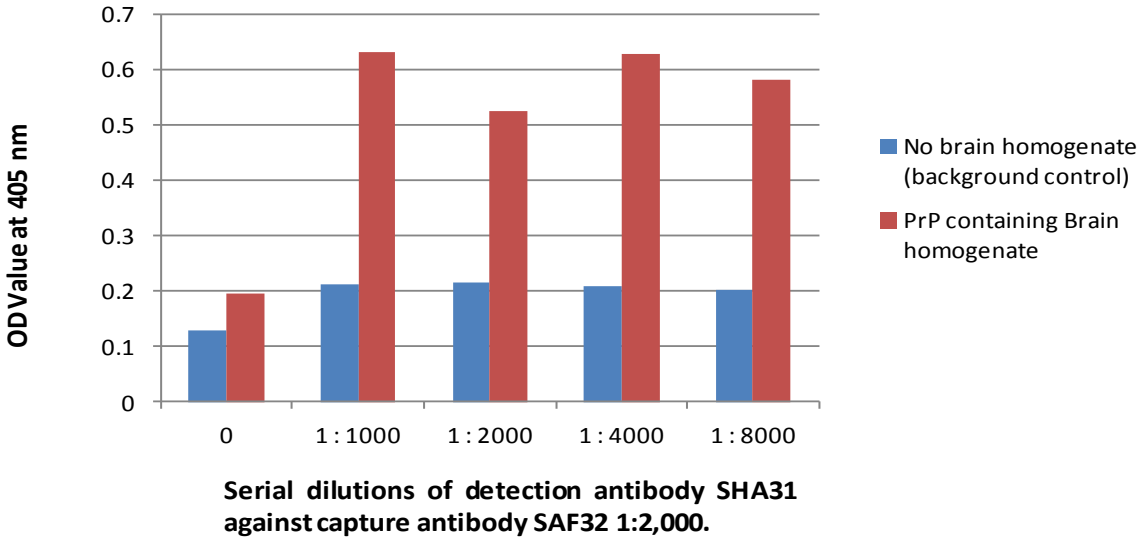


Table 3.1: Determining capture antibody concentration, specificity and compatibility: Dilution series from 1:2,000 to 1:12,000 for a panel of IgG mouse monoclonal capture antibodies SAF15 (A) SAF32 (B), SAF34 (C), and detection antibody SHA31 at a dilution of 1: 8,000. Signals (OD values at 405 nm) generated with 1 µl 10% healthy brain homogenate per well and denatured with SDS were compared. Further controls included omitting capture antibody (no coating) or detection antibody (no SHA31) or no PrP containing sample (-- BH). All samples were added to ELISA wells in triplicate. The average signals obtained for a PrP sample (+ BH) and control background (-- BH) were calculated to obtain the highest signal to background ratio for each antibody at different concentrations (shown in bold). The highest signal to background ratio were obtained with SAF34 (1: 8,000) as a capture antibody together with SHA31 as a detection antibody (B). (This experiment was done once only).

(A) SAF15:

Coating SAF15 in different dilutions	OD value after 60 minutes incubation with the substrate				Signal to background ratio
	+ BH	-- BH	no SHA31	no coating	
1: 2,000	0.4559	0.2189	0.1036	0.0929	2.08
1: 3,000	0.4202	0.1945	0.0948	0.087	2.1
1: 5,000	**0.4175**	**0.1513**	**0.0856**	**0.092**	**2.7**
1: 8,000	**0.3424**	**0.1102**	**0.0759**	**0.0947**	**3.1**
1:12,000	0.1825	0.0669	0.0518	0.0892	0.27
No coating	0.0885	0.0469	0.0436	0.0794	1.8

(B) SAF34:

Coating SAF34 in different dilutions	OD value after 60 minutes incubation with the substrate				Signal to background ratio
	+ BH	-- BH	no SHA31	no coating	
1: 2,000	1.124	0.3292	0.0778	0.072	3.4
1: 3,000	1.1302	0.293	0.074	0.0736	3.8
1: 5,000	**1.1057**	**0.2652**	**0.0697**	**0.0717**	**4.1**
1: 8,000	**0.9478**	**0.2206**	**0.0596**	**0.0702**	**4.2**
1:12,000	**0.5283**	**0.1218**	**0.0507**	**0.07**	**4.3**
No coating	0.0742	0.0444	0.0398	0.0666	1.6

(C) SAF32:

Coating SAF32 in different dilutions	OD value after 60 minutes incubation with the substrate				Signal to background ratio
	+ BH	-- BH	no SHA31	no coating	
1: 2,000	0.7628	0.4261	0.1172	0.0866	1.7
1: 3,000	0.6635	0.2962	0.1136	0.0846	2.2
1: 5,000	0.5057	0.2272	0.094	0.0861	2.2
1: 8,000	**0.4272**	**0.1771**	**0.0758**	**0.0801**	**2.4**
1:12,000	**0.3827**	**0.1269**	**0.0675**	**0.0824**	**3.01**
No coating	0.0802	0.0459	0.039	0.0865	1.7

3.2.2. Optimising blocking agent:

The comparison of different blocking agents demonstrated that incubating the 96 well plate with Ovalbumin for one hour at 20ºC yielded the lowest background values and non-specific binding while SMA had higher background and potential binding of its component to conjugated Alkaline Phosphatase (AP)-Rabbit Anti-Mouse IgG1. There was no difference between using PBS or Tris-buffer on the background of the ELISA (Table 3.2). Further experiments confirmed that using a concentration of 0.5 - 1 % (w/v) Ovalbumin diluted in PBST was the optimum for the lowest background values. Although, bovine serum albumin showed similar advantage to the Ovalbumin (data not shown), the latter blocker was selected for routine use in order to avoid potential cross-reactivity of the selected antibodies with any PrP^{C} protein fragments that may be present in the BSA.

3.2.3. Optimising the denaturation of PrP:

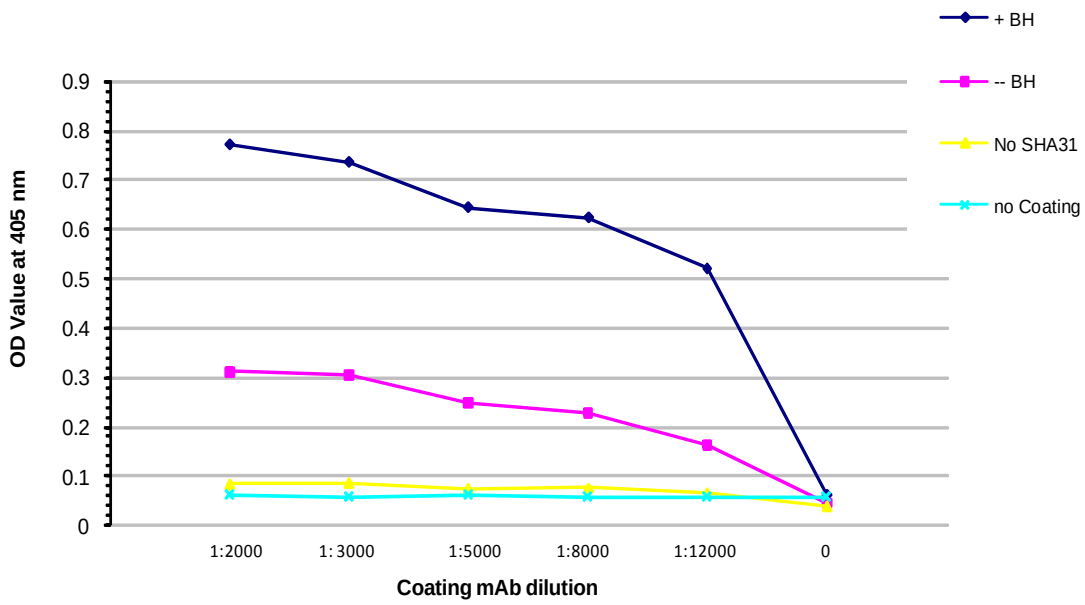
All healthy brain samples were denatured in different denaturation conditions: 1) boiling for 5 minutes, 2) treatment with SDS (Cat No. S1030, Melford, Ipswich, UK) up to a final concentration of 4% (v/v) at 100ºC for 10 minutes, 3) treatment with Guanidine hydrochloride (Gdn-HCl) (Cat No. 4505, Sigma) up to 4% at 80ºC for 5 minutes. The equivalent of 1 µl 10% healthy brain homogenate in PBS (pH 7.4) (w/v), within a final volume of 200 µl PBST was analysed in each ELISA well. The result of different denaturation conditions showed that heating samples at 100ºC for 10 minutes with 4% (w/v) SDS had the optimum epitope exposing effect for the detection antibody SHA31 and capture antibody SAF34 (Graph 3.1).

Table 3.2: Comparing the effect of different blocking reagents and dilution buffers on background signals: an ELISA plate was coated with SAF34 (1: 8,000) as a capture antibody. The plate was divided into different parts and each part was loaded with one blocking solution for one hour. The plate was then loaded with 1 µl 10% healthy brain homogenate per well after denaturation with 4% SDS, and incubated at room temperature for one hour. The bound PrP was detected with SHA31 (1: 8,000) and conjugated Alkaline Phosphates (AP)-Rabbit Anti-Mouse IgG1 (diluted 1: 2,000 in blocking buffer). Signals (OD value) generated from using each blocking agent were compared. Optical density (OD value at 405 nm) for the lowest background was obtained primarily through blocking with Ovalbumin.

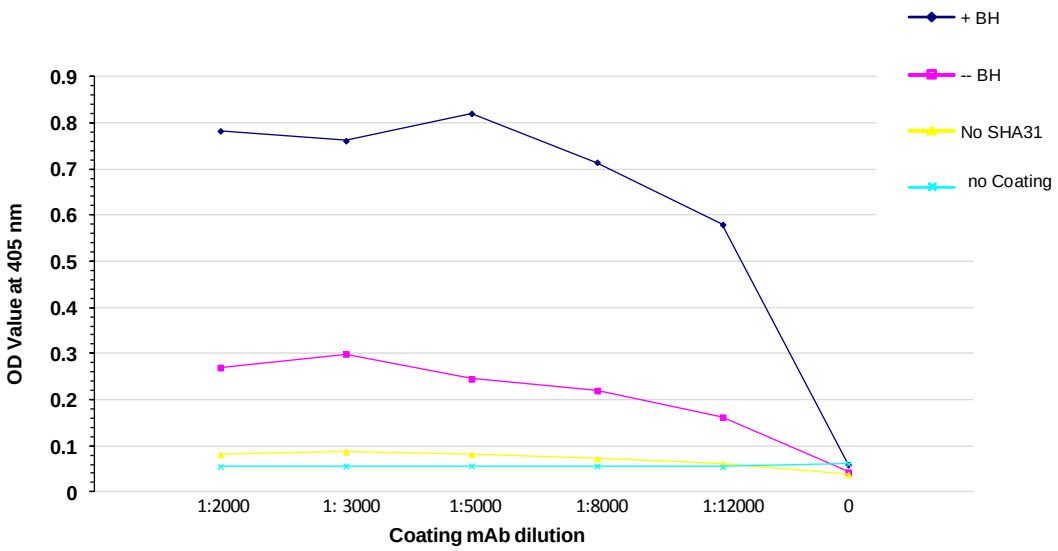
Blocking Solutions	Absorbance at 405 nm for ELISAs employing different buffer systems			
	PBS[c]		Tris buffer[d]	
	PrP	No PrP	PrP	No PrP
3% SMA[a]	2.58065	0.4029	2.5492	0.36085
1% SMA	2.09795	0.1472	1.54365	0.13575
3% Ovalbumin[b]	1.8432	0.14605	2.1881	0.15665
1% Ovalbumin	1.4867	0.12395	1.68035	0.13205

[a] SMA: Powdered milk baby formula
[b]Ovalbumin: Albumin, Chicken Egg
[c]PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, in distilled water to 1 Litre, pH 7
[d]Tris buffer: 8 g NaCl, 0.2 g KCl, 3 g Tris base in distilled water to 1 Litre, pH 8

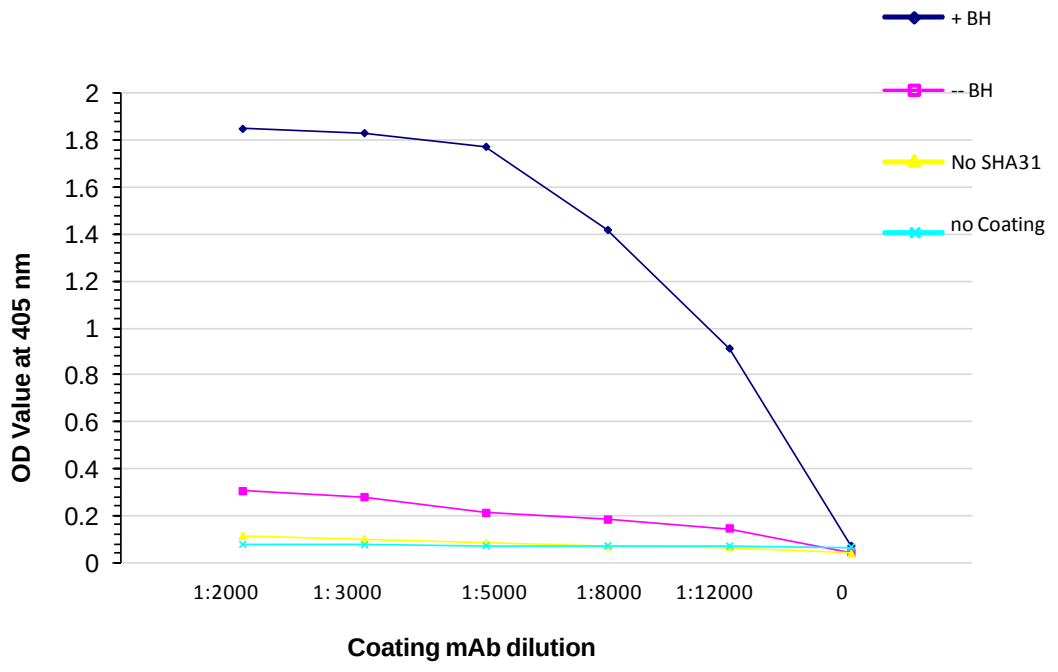
Graph 3.2: Optimising denaturation conditions for PrP: each of four ELISA plates, each was coated with dilution series of SAF34 as capture antibody from 1: 2,000 to 1: 12,000. Each ELISA plate was loaded with 1 µl 10% healthy brain homogenate per well subjected to different denaturation conditions; (A) no denaturation, (B) denaturation through boiling, (C) denaturation with 4% SDS and (D) denaturation with 4% GndHCl. Bound PrP containing brain homogenate was detected with detection antibody SHA31 at a dilution 1: 8,000. Further controls included omitting capture antibody (no coating) or detection antibody (no SHA31) or no PrP containing sample (-- BH). Signals (OD value) generated from each denaturation condition were compared. Optical density (OD value at 405 nm) for the highest detection of 1 µl PrP containing brain homogenate were obtained through denaturing PrP containing samples with 4% SDS.



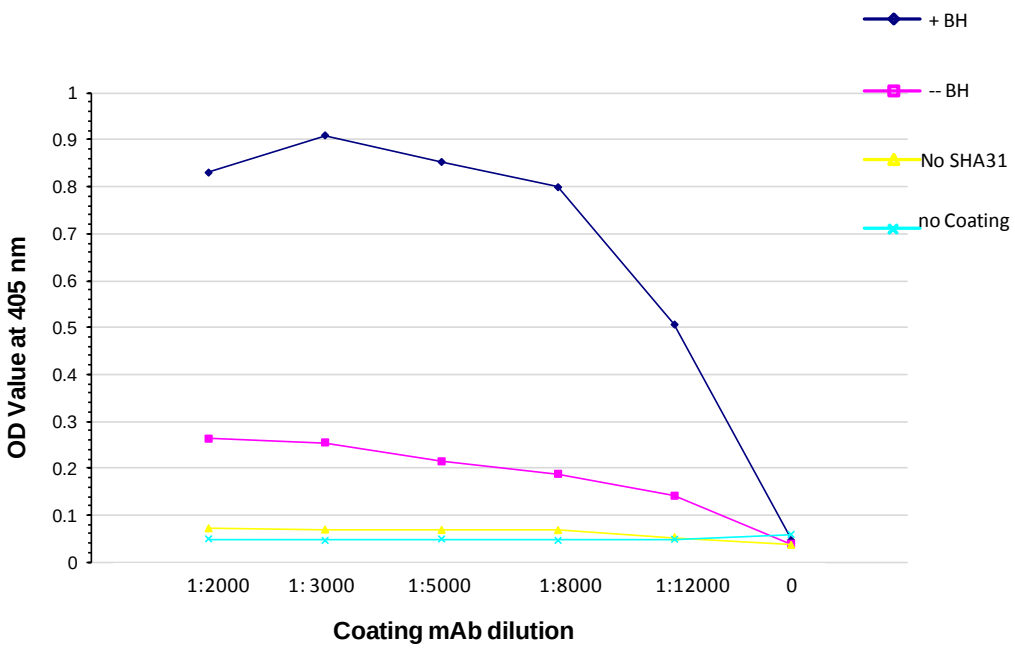
(A) The plate was loaded with 1 µl 10% (w/v) healthy brain homogenate per well without denaturation



(B) The plate was loaded with 1 µl 10% (w/v) healthy brain homogenate per well, denatured through boiling.



(C) The plate was loaded with 1 µl 10% (w/v) healthy brain homogenate per well, denaturation with 4% SDS.



(D) The plate was loaded with 1 µl 10% (w/v) healthy brain homogenate per well, denaturation with 4% GndHCl.

3.2.4. Precipitation of disease-associated PrP:

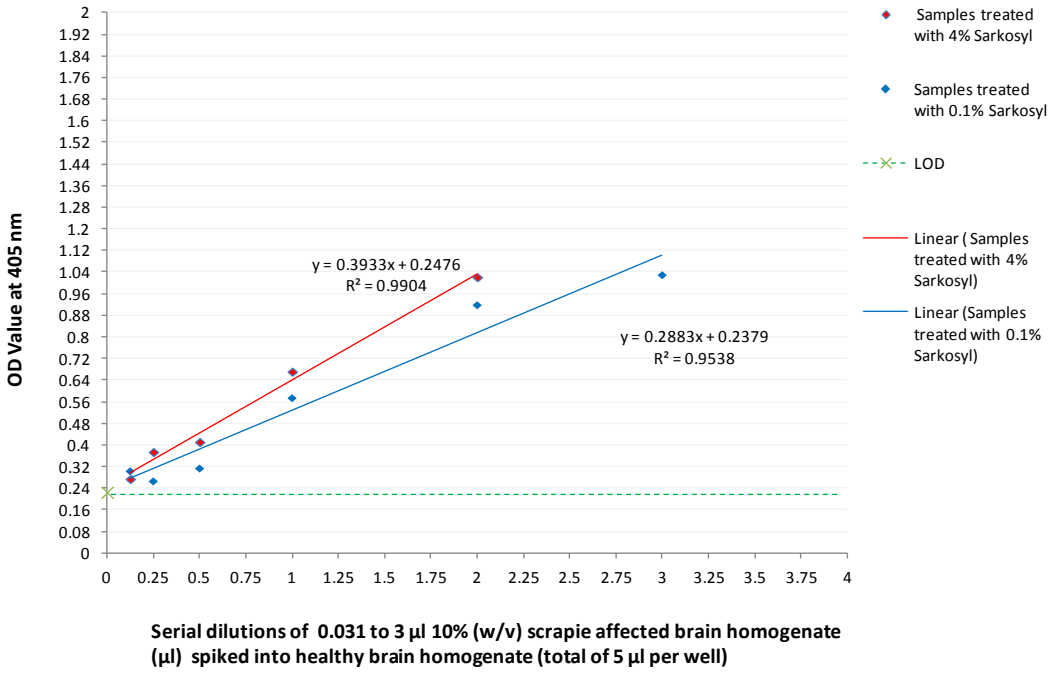
Two series of 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2 µl, 3 µl positive brain homogenates spiked into healthy brain homogenates up to a total of 5 µl of brain homogenates per well were analysed. One dilution series was incubated with an equal amount of PBS containing 0.1% N-lauryl-sarcosyl and the second dilution series with PBS containing 4% N-lauryl-sarcosyl to evaluate the influence of the concentration of N-lauryl-sarcosyl on PrP^{Sc} precipitation. The comparison of these two sets of samples was undertaken to determine the optimum conditions for the precipitation of aggregated PrP^{Sc}. All samples were incubated for 30 minutes at 37ºC and then precipitated with NaPTA. All samples were denatured and detected using the optimised ELISA procedure. The use of 4% Na-lauryl-Sarcosyl, compared to 0.1%, increased PrP^{Sc} recovery by more than 10% (Graph 3.3).

3.2.5. Generation of standard curves for PrP^{Sc}: determination and validation of assay parameters:

Production of PrP^{Sc} standard curves was performed using three dilution series of diseased brain homogenates as follow: Dilution series of 0.1 µl, 0.2 µl, 0.4 µl, 0.6 µl, 0.8 µl 10% positive scrapie brain homogenates (w/v) into healthy brain homogenates making a total of 1 µl brain homogenate per well. Two dilution series of 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2 µl, 3 µl, 4 µl, 5 µl positive brain homogenates; one spiked into healthy brain homogenates up to a total of 5 µl of brain homogenates per well, and the second one spiked into PBS up to a total of 5 µl of per well.

The negative control samples were either healthy brain homogenate or PBS only. All samples were treated with the protease TH, and then PrP^{Sc} precipitated with 4% N-lauryl-sarcosyl and NaPTA. All samples were denatured and detected using the optimised ELISA procedure as detailed in the previous sections. These ELISAs were performed to determine the limit of detection (LOD) for PrP^{Sc} within the context of buffer and when present within brain, representing the LOD for detecting a volume of diseased-brain and for detecting decreasing levels PrP^{Sc} within the brain material respectively.

Graph 3.3: Comparison of two different concentrations of Na-lauryl-sarcosyl (4% and 0.1% respectively) for the precipitation of PrP^{Sc}: Two spiked concentration series of 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2 µl, and 3 µl positive brain homogenates were spiked into healthy brain homogenates to a total volume of 5 µl of brain homogenates per well. The first concentration series was treated with an equal amount of PBS containing 0.1% N-lauryl-sarcosyl and the second one with PBS containing 4% N-lauryl-sarcosyl for 30 minutes at 37ºC to determine the optimum conditions for precipitation of aggregated PrP^{Sc}. The comparison of signals (OD values at 405 nm) for these two sets of samples showed increased PrP^{Sc} recovery by more than 10% when samples were incubated with 4% Na-lauryl-sarcosyl, compared to 0.1%.
LOD= 3* standard deviation of the mean of OD value for negative samples + mean of OD values for healthy brain homogenate (6 replicates)

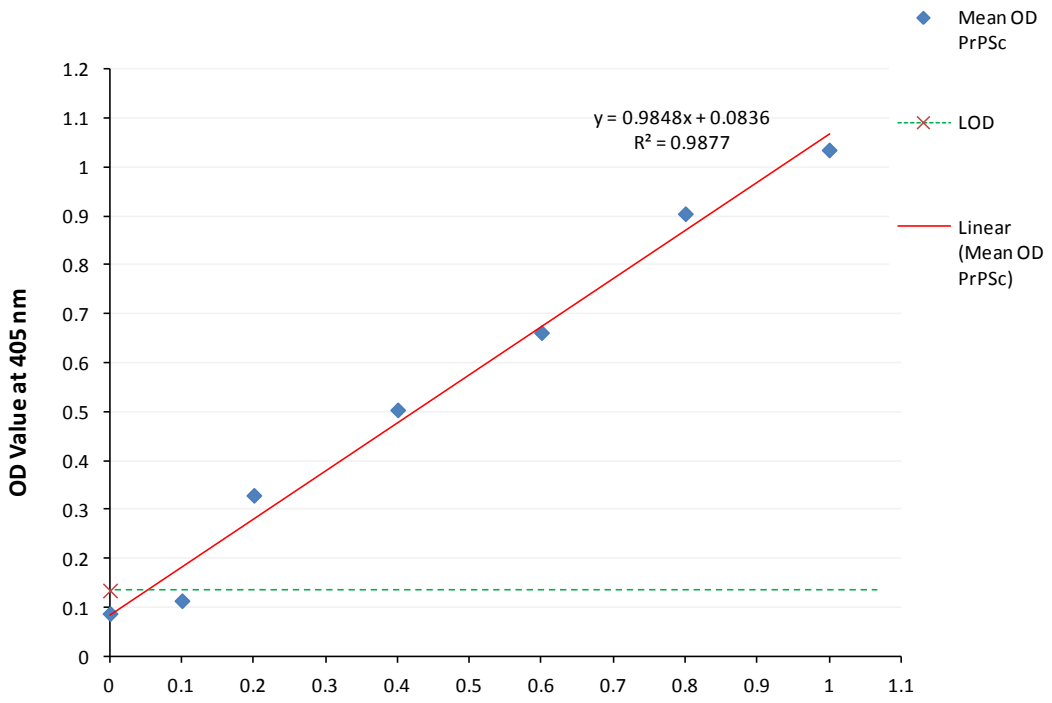


LOD is usually described as the lowest volume of diseased brain material that produces a signal above the background of the assay. This was calculated as the mean OD values produced with PBS or healthy brain homogenate + 3 x standard deviation (stdev) of the mean of OD values of negative samples. R^2 value (The *coefficient of determination*; the square of the sample correlation coefficient between the outcomes and their predicted values) was calculated computationally.

All spiked concentration series into healthy brain homogenates or PBS making a total of 1 µl and 5 µl brain homogenate per well were analysed by the developed ELISA. The result of these experiments showed that both antibodies; SAF34 (1: 8,000) and SHA31 (1: 8,000) have had high compatible detection of VRQ ovine PrP^{Sc} in brain samples. When analysing positive scrapie brain homogenate spiked into healthy brain homogenate at 1 µl in total per well (Graph 3.4: A), as well as 5 µl in total per well (Graph 3.3 and Graph 3.4: B) or into PBS (5 µl total per well, Graph 3.4: C), over 3 experiments, the lowest volume of PrP^{Sc} containing brain homogenate to be detected within the ELISA was 0.125 µl 10% positive brain homogenate (12.5 µg of positive brain material). Linear detection of PrP^{Sc} was up to 3 µl (300 µg of positive brain material) (Graph 3.4: B and C). The R^2 was equal to 0.97 for decreasing amount of PrP^{Sc} within a background of healthy brain homogenate (Graph 3.4: B). Similar result was obtained for decreasing amounts of PrP^{Sc} within a background of PBS ($R^2 = 0.95$) (Graph 3.4: C). The inter-assay variation coefficient (CV; coefficient of variation) was equal to 0.02 (i.e. 2%). These optimised conditions were used in all subsequent ELISA experiments.

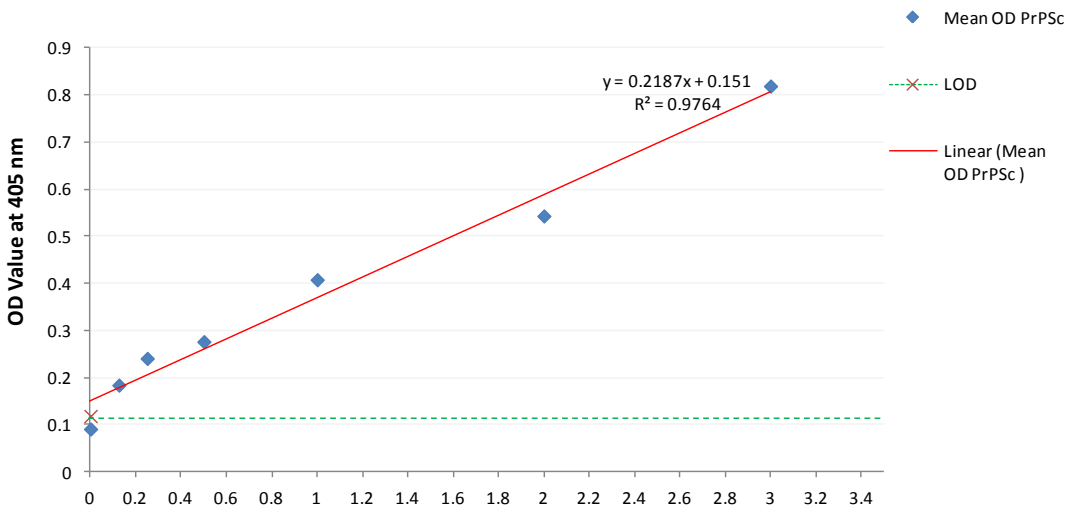
Graph 3.4: Detection of dilution series of positive scrapie brain homogenate: (A) Dilution series of positive scrapie brain homogenate: 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl positive brain homogenates spiked into healthy brain homogenates up to a total of 1 µl of brain homogenates per well. **(B)** & **(C)** Dilution series of Positive Scrapie Brain Homogenate: 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl , 2 µl, 3 µl positive brain homogenates spiked into healthy brain homogenates (B) or PBS (C) up to a total of 5 µl of brain homogenates and/or PBS per well. The negative control samples were either healthy brain homogenate or PBS only .This experiment was performed based on the optimised ELISA procedures to determine the detection limit for PrP^{Sc} when present within brain. All samples were analysed in triplicate and the means of PrP^{Sc} were plotted. After calculating the R^2 (correlation coefficient) over several experiments, the limit of detection of PrP^{Sc} containing brain homogenate within the ELISA was 0.125 µl 10% positive brain homogenate (12.5 µg), with linear detection of PrP^{Sc} up to 3 µl (300 µg of positive brain material).
LOD= 3* standard deviation of the mean of OD value for negative samples + mean of OD values for healthy brain homogenate (6 replicates)

(A)



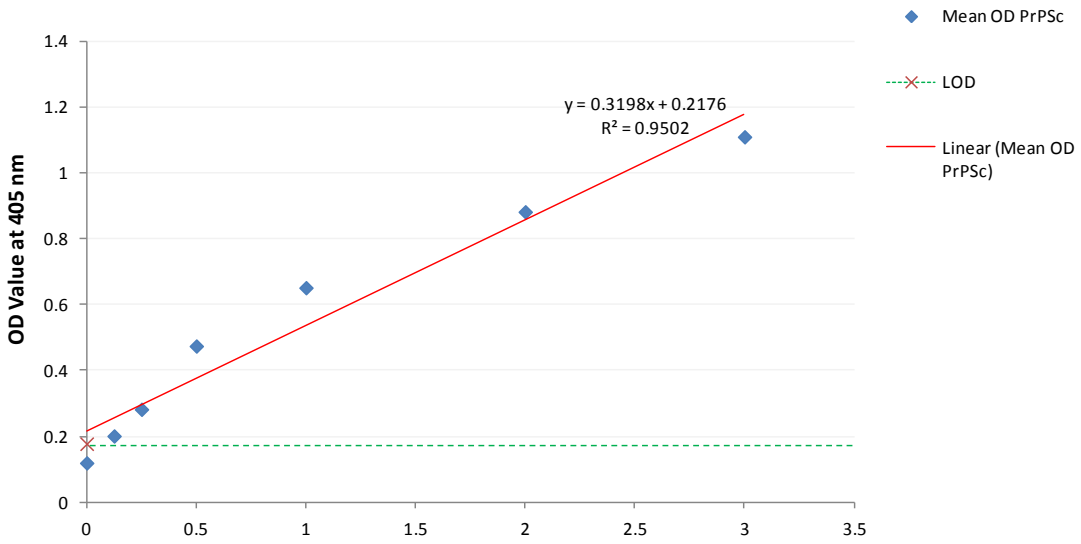
(A) Serial dilutions of 0.031 to 1 µl 10% (w/v) scrapie affected brain homogenate (µl) spiked into healthy brain homogenate (total of 1 µl per well).

(B)



(B) Serial dilutions of 0.031 to 3 µl 10% (w/v) scrapie affected brain homogenate (µl) spiked into healthy brain homogenate (total of 5 µl per well)

(C)



(C) Serial dilutions of 0.031 to 3 µl 10% (w/v) scrapie affected brain homogenate spiked into PBS (total of 5 µl scrapie brain homogenate and PBS per well)

3.2.6. Detection of ovine rPrP:

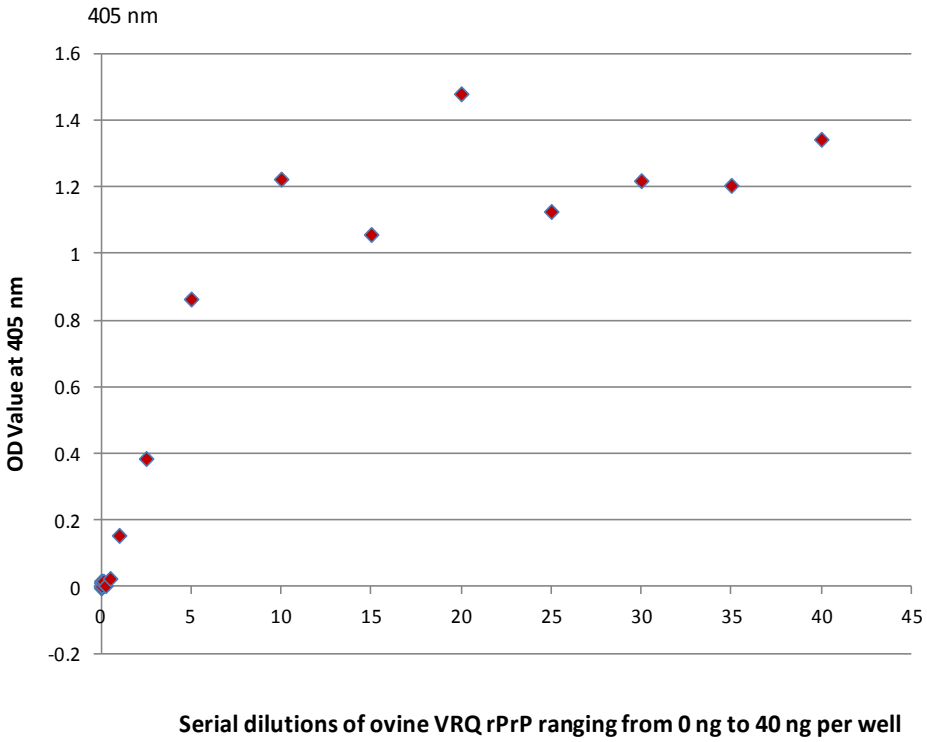
Serially diluted samples containing 0 – 40 ng VRQ rPrP were denatured and the optimised ELISA procedures were carried out. The linear range for the detection of VRQ ovine rPrP was between 0.5 - 5 ng per well (5 - 25 ng/ml) and the R^2 value was equal to 0.992 (Graph 3.5: A & B).

3.2.7. Comparison of PrP detection limits using high sensitivity Western blotting and the developed ELISA protocol:

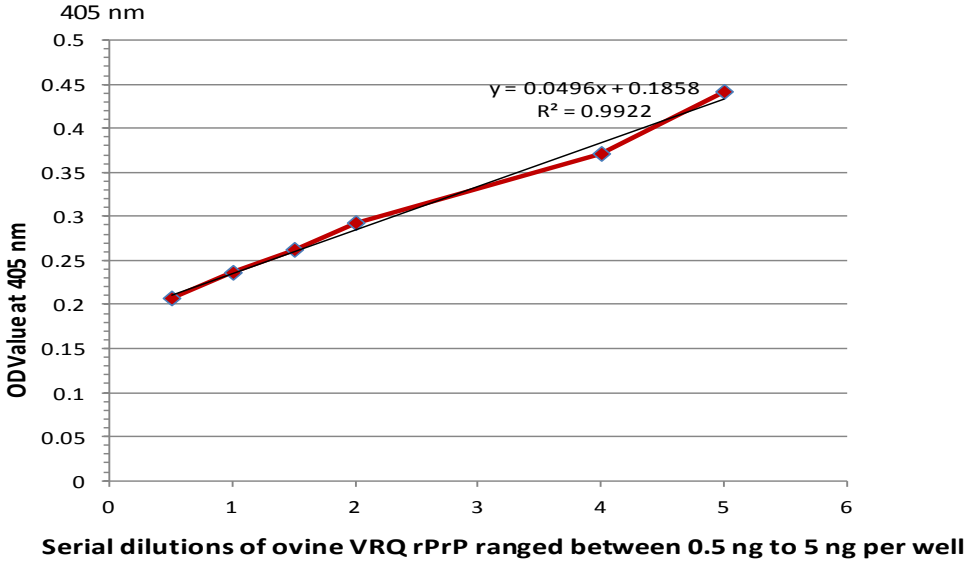
PrP^{Sc} detection by high sensitivity Western blot was carried out using the same samples analysed previously by ELISA (section 3.2.5). Two dilution series of 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2 µl, and 3 µl positive brain homogenates were spiked into healthy brain homogenates to a total volume of 5 µl of brain homogenates per well. The first spiked concentration series was digested with TH (100 µg/ml) at 70ºC for one hour, precipitated with NaPTA and re-suspended in the original sample volume. The second spiked concentration series was digested with PK (50 µg/ml) at 37ºC for one hour. Digested 10% (w/v) brain homogenate samples (5 µl each) were analysed by Western blot. The detection limit of PrP^{Sc} by the developed ELISA assay showed similar PrP^{Sc} detection limits when compared to Western blot analysis; the latter detected the equivalent of 0.062 to 0.125 µl of positive 10% (w/v) brain homogenate (Figure 3.1: A, B & C). It was noticeable that the control sample (healthy brain homogenate) was not fully digested with PK (Figure 3.1: A) whilst it was fully removed when treated with TH and precipitated with NaPTA (Figure 3.1 B & C). These data indicate that both TH digestion and the process of precipitation with N-lauryl-sarcosyl and NaPTA may aid in removing PrP^{C} compared to PK digestion alone or that higher doses of PK are needed to remove PrP^{C} from these samples. Furthermore, when using brain homogenate materials from healthy sheep (VRQ/VRQ, cerebellum) and scrapie infected sheep (VRQ/VRQ, brain stem), the developed ELISA showed similar sensitivity for PrP^{Sc} detection as an established high-sensitivity Western blot technique, routinely applied for the detection of PrP^{Sc}.

Graph 3.5: Detection of dilution series of ovine VRQ rPrP: Dilution series of ovine VRQ rPrP ranging from 0 ng to 40 ng were loaded in triplicate into an ELISA plate coated with SAF34 (1: 8,000). Bound rPrP was detected with SHA31 (1: 8,000) and conjugated Alkaline Phosphates (AP)-Rabbit Anti-Mouse IgG1 (diluted 1: 2,000 in blocking buffer) (A). Determining the R^2 value showed that the linear range for the detection of ovine VRQ rPrP ranged between 0.5 - 5 ng per well (5 - 25 ng/ml) (B).

(A)



(B)



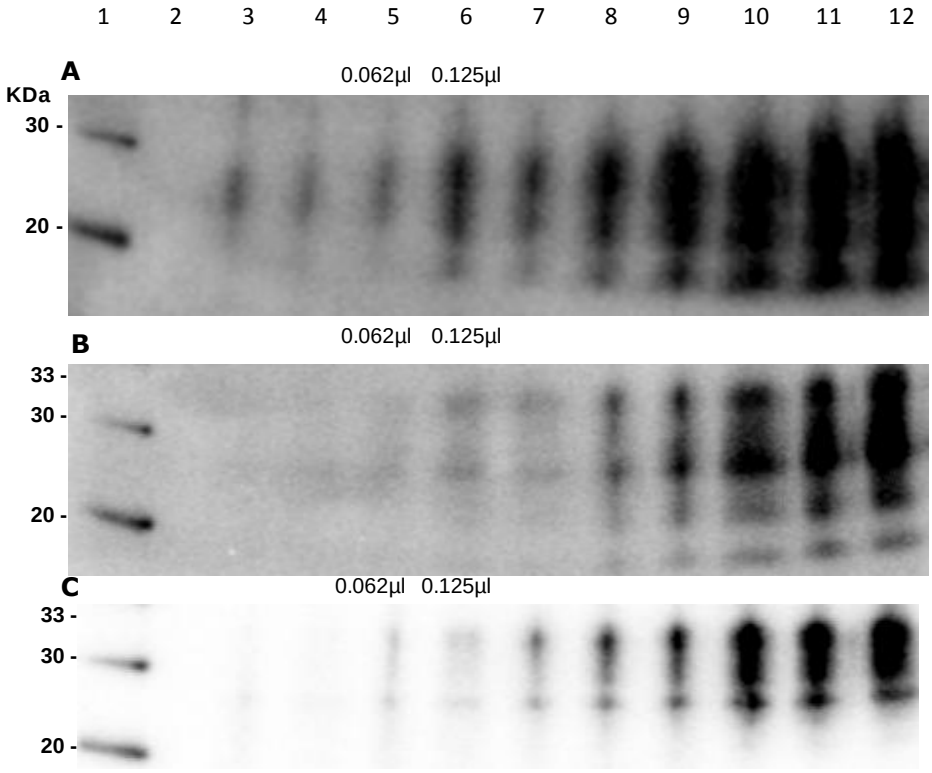


Figure 3.1: PrP detection limits using Western blot after PK and TH digestion: Two dilution series of 0, 0.031 µl, 0.062 µl, 0.125 µl, 0.25 µl, 0.5 µl, 1 µl, 2.5 µl, 2 µl, and 3 µl 10% positive brain homogenate were spiked into healthy brain homogenates to a total volume of 5 µl (lanes 3-12). One dilution series was digested with PK (50 µg/ml) at 37ºC for one hour (A). The other two series were digested with TH (100 µg/ml) at 70ºC for one hour, and then processed based on the optimised procedures for NaPTA precipitation of PrP^{Sc} and re-suspension of samples in the original volumes (B & C). Digested samples (5 µl each) were loaded per well. Molecular weight markers are shown (Lane 1) and PrP was visualised by probing with monoclonal antibodies SHA31 (PrP epitope: 145 – 152) (A and B) or SAF34 (PrP epitope: 59 – 89) (C) and detection with Western breeze kit.

3.2.8. Comparison between high sensitivity Western blotting and the developed ELISA protocol for detecting PMCA-PrP^{Sc}:

The detection of PMCA-PrP^{Sc} samples (products of PMCA amplification of PrP^{Sc} seed) from a range of TSE affected sheep were analysed by the developed ELISA and an established Western blot protocol. Samples spiked into homozygous ARQ substrate were analysed (in triplicates) and detected by the developed ELISA before amplification (Time 0) and after one round of PMCA (Time 24). The signals obtained were qualititatively assessed; i.e. samples were judged to be successfully amplified based on the difference in OD values before and after amplification (Graph 3.6).

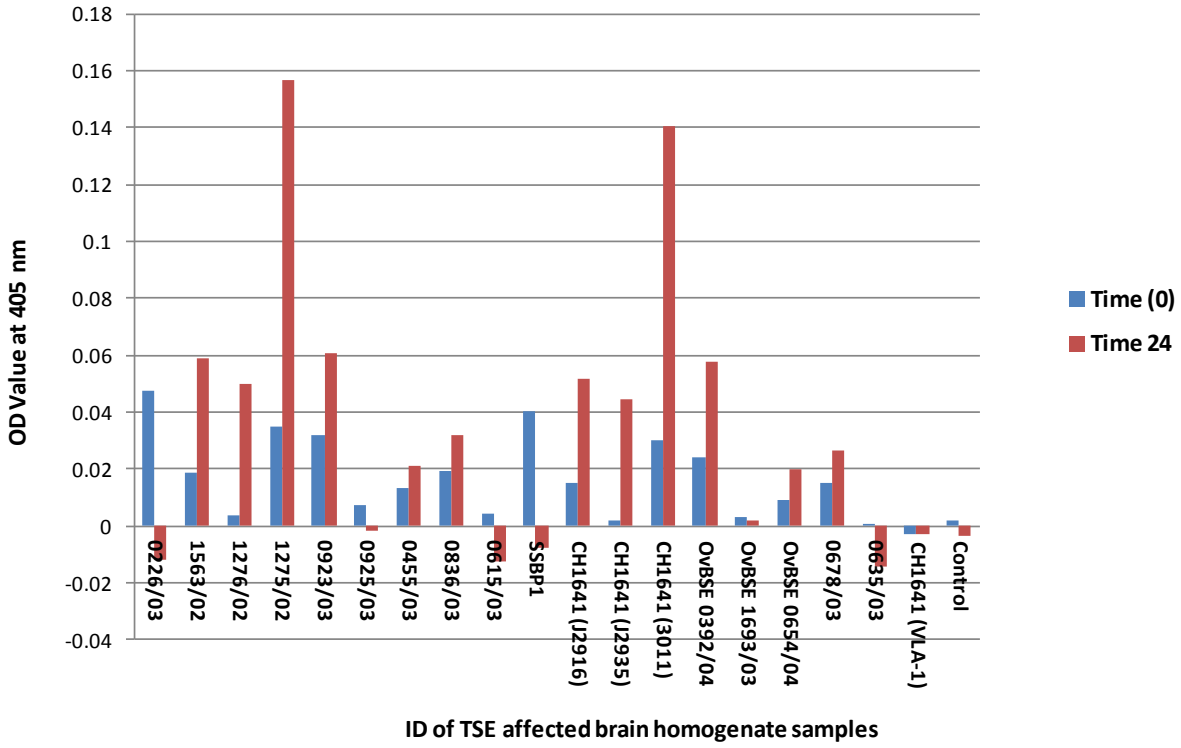
Some PMCA-PrP^{Sc} samples cannot be detected by ELISA whilst they can be detected by Western blotting using the same detection antibody SHA31 (Figure 3.2 and Table 3.3)

In 0925/03 and SSBP1 isolates (Figure 3.2: panel B, lanes 2 and 10 respectively), Western blot analysis showed that PMCA-PrP^{Sc} is composed mainly of truncated C-terminal fragments, lacking the binding epitope of the capture antibody of the ELISA. Since the developed ELISA assay detects full length PrP^{Sc} through initial binding to a capture antibody to the N- terminal domain, these two isolates were not captured and could not be detected.

However, a lack of full length PMCA-PrP^{Sc} does not fully explain the differences in detection between ELISA and Western blotting. For example, the sample ov BSE 0654 and sample 0678 (Figure 3.2: panel D, lanes 2 and 4 and Table 3.3) gave high signal level of TH resistant full length PMCA-PrP^{Sc} in Western blot but were still not detected in the ELISA.

In classical scrapie 1276/02 (VRQ/VRQ) (Figure 3.2: panel A, lanes 5 and 6), 0615/03 (ARQ/VRQ) (Figure 3.2: panel B, lanes 7 and 8 respectively) and 0635/03 (ARQ/ARQ) (Figure 3.2: panel D, lanes 5 and 6) isolates, it seems that PMCA-PrP^{Sc} product contained conformers that are more sensitive to treatment with TH and more resistant to PK. This was demonstrated in the stronger signal intensity for PK PrP^{res} than TH PrP^{res} of the same samples. Since these samples were classical scrapie of different genotypes, it seems unlikely strain or genotype dictates the observed variability towards proteases.

Graph 3.6: Detection of a PMCA-PrPSc by the developed ELISA: 1 µl 10% (w/v) cerebellum homogenate of different TSE samples were spiked into homozygous ARQ substrate to a final volume 100 µl in duplicate. First set of samples were kept at - 20ºC (Time 0). The second set was subjected to one round of PMCA reaction (Time 24). 5µl of each sample in both sets were digested with TH and analysed by the developed ELISA in triplicates (Chapter 2: Materials and methods; 2.7.8). OD value (absorbance read at 405 nm) for each sample was reported and analysed qualitatively. Samples were recorded as detected or not detected based on the difference of OD values before (Time 0) and after amplification (Time 24) for each sample.



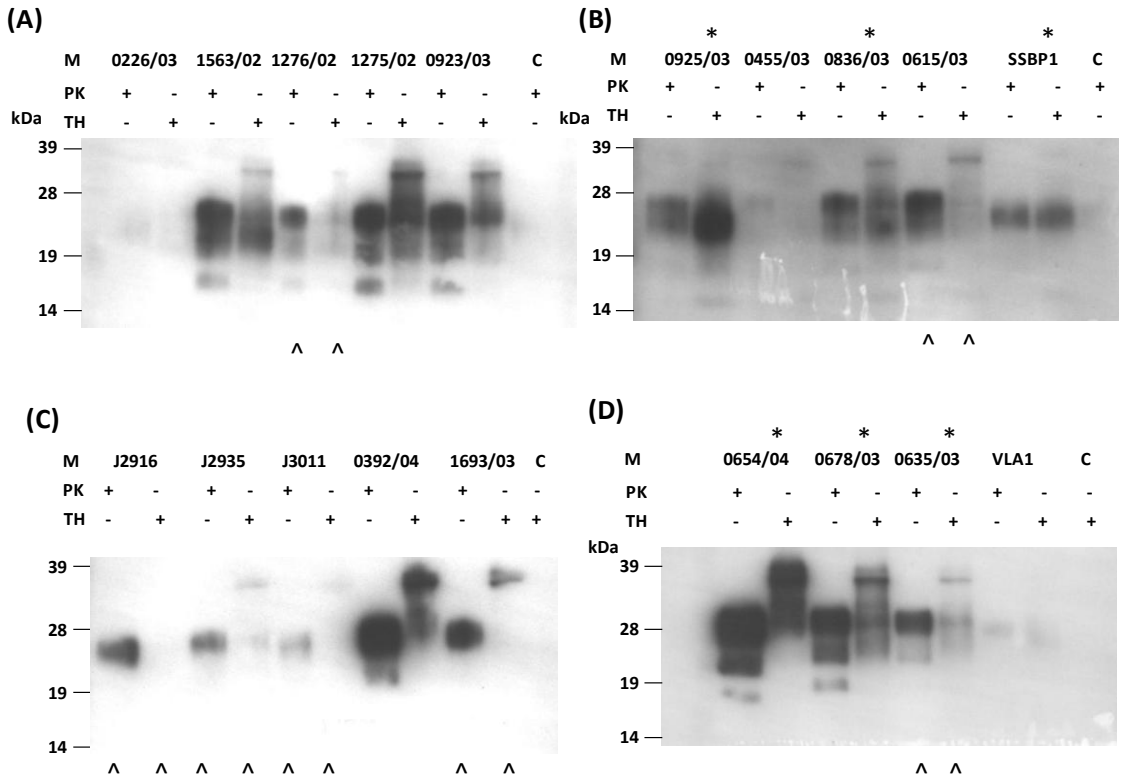


Figure 3.2: Comparison between the developed ELISA protocol and Western blotting to detect protease resistant PMCA- PrP^{Sc}: 1 µl cerebellum homogenate of different TSE samples were spiked into ARQ substrate to a final volume 100 µl. The mixture was subjected to 1 round of sPMCA reaction as described. After completing the reaction, 20 µl of each sonicated sample was digested with PK (50 µg/ml) for 1 hour at 37ºC. Further 20 µl of each sonicated sample was digested for one hour with TH at 70ºC. 5 µl of each digested sample was analyzed by Western blotting and probed with SHA31 antibody. Each lane is marked with the type of protease used for digesting the sample. "C" is ARQ substrate (control non-spiked sample subjected to sonication). Sample details are given in table 3.3. Samples detected by Western blot that were not detected by ELISA are indicated by (*). Samples that showed PK^{res} TH^{sen} conformers after amplification are indicated by (^)

Table 3.3: Comparison between Western blotting and the developed ELISA protocol for detecting PMCA- PrP^{Sc}:

Lane No.	Strain	Isolate ID	Strain Genotype	Protease used for digestion	ELISA result (D, ND)[1]	Western Blotting signal evaluation (W, M, S)[2]
Membrane A						
1	Classical Scrapie	0226/03	ARQ/VRQ	PK		ND
2	Classical Scrapie	0226/03	ARQ/VRQ	TH	ND	ND
3	Classical Scrapie	1563/02	VRQ/VRQ	PK		S
4	Classical Scrapie	1563/02	VRQ/VRQ	TH	D	S
5	Classical Scrapie	1276/02	VRQ/VRQ	PK		M
6	Classical Scrapie	1276/02	VRQ/VRQ	TH	D	W/ND
7	Classical Scrapie	1275/02	VRQ/VRQ	PK		S
8	Classical Scrapie	1275/02	VRQ/VRQ	TH	D	S
9	Classical Scrapie	0923/03	VRQ/VRQ	PK		S
10	Classical Scrapie	0923/03	VRQ/VRQ	TH	D	M
Membrane B						
1	Classical Scrapie	0925/03	VRQ/VRQ	PK		M
2	Classical Scrapie	0925/03	VRQ/VRQ	TH	ND	S*
3	Classical Scrapie	0455/03	ARQ/VRQ	PK		ND
4	Classical Scrapie	0455/03	ARQ/VRQ	TH	ND	ND
5	Classical Scrapie	0836/03	VRQ/VRQ	PK		M
6	Classical Scrapie	0836/03	VRQ/VRQ	TH	ND	M*
7	Classical Scrapie	0615/03	ARQ/VRQ	PK		S
8	Classical Scrapie	0615/03	ARQ/VRQ	TH	ND	W/ND
9	Classical Scrapie- SSBP1	SSBP1	VRQ/VRQ	PK		M
10	Classical Scrapie- SSBP1	SSBP1	VRQ/VRQ	TH	ND	M*
Membrane C						
	CH1641	J2916	ARQ/AHQ	PK		M
	CH1641	J2916	ARQ/AHQ	TH	D	ND
	CH1641	J2935	ARQ/AHQ	PK		M

	CH1641	J2935	ARQ/AHQ	TH	D	W/ND
	CH1641	J3011	AHQ/AHQ	PK		W
	CH1641	J3011	AHQ/AHQ	TH	D	W/ND
	Ov BSE	0392/04	ARQ/ARQ	PK		S
	Ov BSE	0392/04	ARQ/ARQ	TH	D	S/M
	Ov BSE	1693/03	ARQ/ARQ	PK		S
	Ov BSE	1693/03	ARQ/ARQ	TH	ND	ND
	Membrane D					
1	Ov BSE	0654/04	ARQ/ARQ	PK		S
2	Ov BSE	0654/04	ARQ/ARQ	TH	ND	S*
3	Classical Scrapie	0678/03	ARQ/ARQ	PK		S
4	Classical Scrapie	0678/03	ARQ/ARQ	TH	ND	M*
5	Classical Scrapie	0635/03	ARQ/ARQ	PK		S
6	Classical Scrapie	0635/03	ARQ/ARQ	TH	ND	W
7	CH1641	VLA-1	AHQ/AHQ	PK		W/ND
8	CH1641	VLA-1	AHQ/AHQ	TH	ND	W/ND
	Control (C; healthy brain homogenate)	1035/05	ARQ/ARQ	PK		ND
	Control (C; healthy brain homogenate)	1035/05	ARQ/ARQ	TH	ND	ND

[1]D: detected, ND: not detected

[2] W: Weak signal, M: medium signal, S: strong signal

*samples detected by Western blot and were not detected by ELISA.

3.3. Discussion:

The development of high throughput immunoassays such as ELISAs to detect PrP^{Sc}, the surrogate marker for TSE diseases (Prusiner 1998), has received great efforts from many research groups and companies. Different approaches have been taken aiming at increasing the sensitivity and robustness of such assays either through concentrating PrP^{Sc} aggregates or increasing its concentration through in vitro amplification or both (Chang *et al*., 2007). Although many trials were successful the requirement for a highly specific, sensitive and very strict analytical diagnostic method for routine early diagnosis of prion diseases has not been fulfilled (Grassi *et al*., 2008).

I developed an ELISA for quantifying PrP^{Sc} in prion infected brain tissue, as well as detecting PrP^{Sc} following *in vitro* prion amplification. The assay uses isotype specificity to recognise a detection antibody directed against the PrP^{Sc} core above a capture antibody directed towards an epitope in the N-terminal part of PrP^{Sc}. This was achieved through treating samples with the protease TH (Owen *et al*., 2007), thus digesting PrP^{C} and revealing full length PrP^{Sc}, precipitating the resulting PrP^{Sc} with NaPTA (Cronier *et al*., 2008) to isolate this isoform away from PrP^{C} fragments and then detecting the PrP^{Sc} within the sandwich ELISA. The sensitivity and similarity in LOD (12.5 µg of positive brain material) between dilution series of PrP^{Sc} spiked in PBS and healthy brain homogenate prove the high specificity of the developed ELISA to detect low level of PrP^{Sc} in brain samples.

Although, the developed ELISA assay showed similar PrP^{Sc} detection limits in classical scrapie diseased brain homogenates when compared to Western blot analysis, I could not detect some PMCA-PrP^{Sc} in PMCA propagated samples using the same assay. This result could not be considered a limitation to the sensitivity of the assay. More likely it shows the biochemical difference of some PMCA-PrP^{Sc} compared to the original PrP^{Sc} seed. For example, Western blot analysis of classical scrapie affected cerebellum isolate (0635/03) did not show any difference between PK and TH resistant PrP^{Sc} (Chapter 4: Molecular differences of experimental and natural strains/isolates of ovine prion diseases, Figure 4.3, Panel C). On the contrary, PMCA amplified products from the same sample; 0635/03-PMCA-PrP^{Sc} showed more resistance to PK than TH. Therefore, the observed discrepancy between

Western blot and ELISA detection of the amount of resistant PMCA-PrP^{Sc} might reflect the variation of PMCA amplified isoforms which could be related to the conformers contained within the original sample and/or the effect of sonication on the propagation of distinct conformers. However, such observation needs to be further investigated.

Detecting PrP^{Sc} by the developed ELISA technique has several advantages over Western blotting; first, considering the variation of PrP^{Sc} concentration in test samples, the methodology allows comparison to a rPrP standard curve within each assay, facilitating the quantitation of PrP^{Sc} (Maddison *et al*., 2010). Second, large numbers of experimental samples can be analyzed at one time. Third, since the developed ELISA detects only full length PrP^{Sc}, it can be used with other commercial ELISAs (e.g. Bio-Rad® TeSeE Sheep/Goat ELISA) to compare the proportion of N-terminally truncated to full length PrP^{Sc} in samples or the influence of other factors on the processing of PrP^{Sc} and the production of truncated PrP^{Sc} fragments (Maddison *et al*., 2012). Fourth, the use of the protease TH has the potential of detecting PK-sensitive PrP^{Sc} (Cronier *et al*., 2008) potentially making this assay more sensitive for some prion isolates. Fifth, it is shown in this study that PK, when used under standard conditions routinely applied for PrP^{Sc} detection (50 µg/ml), might not fully digest all healthy PrP on every occasion (as shown in the result section, 3.2.7 and figure 3.1 A), an outcome reported previously (Hope, *et al*., 1999 and TSE EU Community Reference Laboratory, 2007). Therefore, digestion with TH can overcome this problem to differentiate between healthy and scrapie affected animals.

Chapter 4: Molecular Differences of Experimental and Natural Strains/Isolates of Ovine Prion Diseases

4.1. Introduction:

According to the protein-only hypothesis, PrP^{Sc} is the main marker for prion diseases (Prusiner, 1998 & 1991) and specific prion diseases have been found to differentiate into multiple distinct strains. Scrapie in sheep has exhibited diverse heterogeneous prion strains with significant differences in the length of incubation periods and lesion profiles after transmission into transgenic mice or susceptible sheep (Bruce, 1993).

Mouse bioassay (either inbred wild-type mouse or transgenic mouse) is the definitive strain typing method. The length of incubation period, reproducible patterns of vacuolation and intensity of vacuolation in selected neuroanatomic locations are particular fearture of each strain/infected mouse line. These specific criteria allow successful discrimination between strains (Beck *et al*., 2010, Gavier-Widén, *et al*., 2005).

Histopathological changes and IHC analysis provides a tool to distinguish between distinct prion strains by detection of disease-related PrP species. Strains are defined through differences in neuropathological targeting and abundance of disease-related PrP in brain tissue (Jeffrey *et al*., 2006a, Gonzalez *et al*., 2002, van Keulen *et al*., 1996) and vacuolation profiles (Begara-McGorum *et al*., 2002, Ligios *et al*., 2002). Furthermore, the IHC approach of PrP^{d} (disease-specific prion protein) epitope mapping based on the intracellular cleavage of PrP^{d} together with immunoreactivity of PrP^{d} to a standard panel of PrP antibodies that recognise different epitopes allowed differentiation between SSBP/1 scrapie and CH1641 scrapie in sheep (Jeffrey and Gonzalez, 2007).

In addition, differences in the biochemical characterization of different scrapie isolates by Western blotting through variability in glycoprofiles, antibody reactivity and the molecular weight profiles of PrP^{Sc} fragments after PK and TH digestion have added valuable rapid tests to discriminate between classical scrapie and experimental ovine BSE (Baron and Biacabe, 2007, Owen *et al*., 2007, Thuring *et al*., 2004, Stack *et al*., 2002). However, migration pattern differences of PrP^{res} in Western blots showed that PK PrP^{res} from sheep inoculated with CH1641 scrapie gave a molecular profile similar to that of sheep inoculated with BSE (Lezmi *et al*., 2004, Stack *et al*., 2002, Hope *et al*., 1999). Although, both strains could be differentiated through the more protracted method of IHC PrP^{d} profiling and peptide mapping (Jeffrey *et al*.,

2006), the use of established rapid strain typing tests raises the possibility that field scrapie strains similar to CH1641 might be misclassified as ovine BSE.

Strain discrimination is of keen interest for prion diagnostics and for public health. This study extends previous reports (Stack *et al*., 2006, Thuring *et al*., 2004) using Western blotting of cerebellum homogenates for the diagnosis and strain typing of a suspected case of TSE in sheep. These data also provide more information towards our understanding of the sensitivity and resistance of PrP^{Sc} towards proteases.

4.2. Results:

4.2.1. Molecular profiles of PrP^{res} fragments:

Representative results of Western blotting analysis of PrP^{res} from the panel of different experimental TSE strains (Table 4.1) are shown from two experimental repeats. Samples were examined for the electrophoretic mobility and antibody binding between PK and TH resistant PrP^{res} in experimental CH1641, SSBP1 and ovine BSE infected cerebellum tissue. Not all PK digested samples have shown the characteristic three glycoform banding patterns of PrP^{Sc}; diglycosylated, monoglycosylated and unglycosylated bands. There were also obvious variations in the levels of PrP^{res} among individual isolates within each group of samples representing a particular strain, i.e. the samples from 4 x CH1641-experimentally infected sheep and 3 x BSE experimentally infected sheep (Figure 4.1).

For samples probed with L42 (epitope 148-YEDRYY-153) and SAF84 antibodies (epitope 166-RPVDQY-172): the 3 PrP^{res} glycoforms of PK digested Ov BSE (0392/04) (Figure 4.1: panels A & C, lane 5, panels B & D, lane 19) and CH1641 (J2935) (Figure 4.1: panels A & C, lane 9 and panels B and D lanes 13) showed a higher molecular weight for non glycosylated PrP^{res} for the Ov BSE sample compared to CH1641. CH1641 (J3011) (Figure 4.1: panels B and D lane 15) gave consistent results for this strain.

Table 4.1: Brain tissues from healthy, experimental BSE and scrapie infected sheep.

Isolate	Strain	Breed	Genotype	Brain region
1035	Healthy	Dorset Horn	ARQ/ARQ	Cer
	SSPB1	Cheviot	VRQ/VRQ	Cer
J2916	CH1641	Cheviot	ARQ/AHQ	Cer
J2935	CH1641	Cheviot	ARQ/AHQ	Cer
J3011	CH1641	Cheviot	AHQ/AHQ	Cer
VLA1	CH1641	Cheviot	AHQ/AHQ	Cer
1693/03	Ovine BSE	Romney	ARQ/ARQ	Cer
0392/04	Ovine BSE	Romney	ARQ/ARQ	Cer
0654/04	Ovine BSE	Romney	ARQ/ARQ	Cer
0226/03	Scrapie	Swaledale	ARQ/VRQ	Cer, CM and SC
0678/03	Scrapie	Suffolk cross	ARQ/ARQ	Cer and CM
0284/97	Scrapie	Finn Dorset	AHQ/AHQ	Cer, CM and SC
1776/02	Scrapie	Welsh Hill Speckled	VRQ/VRQ	CM
0575/00	Scrapie	Cambridge	ARQ/ARQ	CM
0455/03	Scrapie	Swaledale	ARQ/VRQ	Cer, CM and SC
0635/03	Scrapie	Charollais Cross	ARQ/ARQ	Cer
0615/03	Scrapie	Swaledale	ARQ/VRQ	Cer, CM and SC
1275/02	Scrapie	Welsh Mountain	VRQ/VRQ	Cer, CM and SC
1276/02	Scrapie	Welsh Mountain	VRQ/VRQ	Cer, CM and SC

Brain tissues from healthy, experimental BSE and scrapie infected sheep were obtained from the Animal Health Veterinary Laboratories Agency TSE-Archive (AHVLA; Addlestone, Surrey, United Kingdom). CH1641 & SSBP1 were kindly supplied by N. Hunter (Institute for Animal Health, Neuropathogenesis Unit, Edinburgh, United Kingdom). SSBP1 was passaged at least 24 times through Cheviot and Cheviot crosses, including a final passage in VRQ/VRQ Cheviot sheep at the VLA. CH1641 (animals J2916, J2935 and J3011) were obtained from the Roslin Institute or passaged in AHQ/AHQ sheep at the AHVLA (animal VLA1). Samples were homogenised to 10% brain homogenates; Cerebellum (Cer), Caudal Medulla (CM) and Spinal Cord (SC) (according to the methodology of 2.1. Brain tissue homogenate preparation).

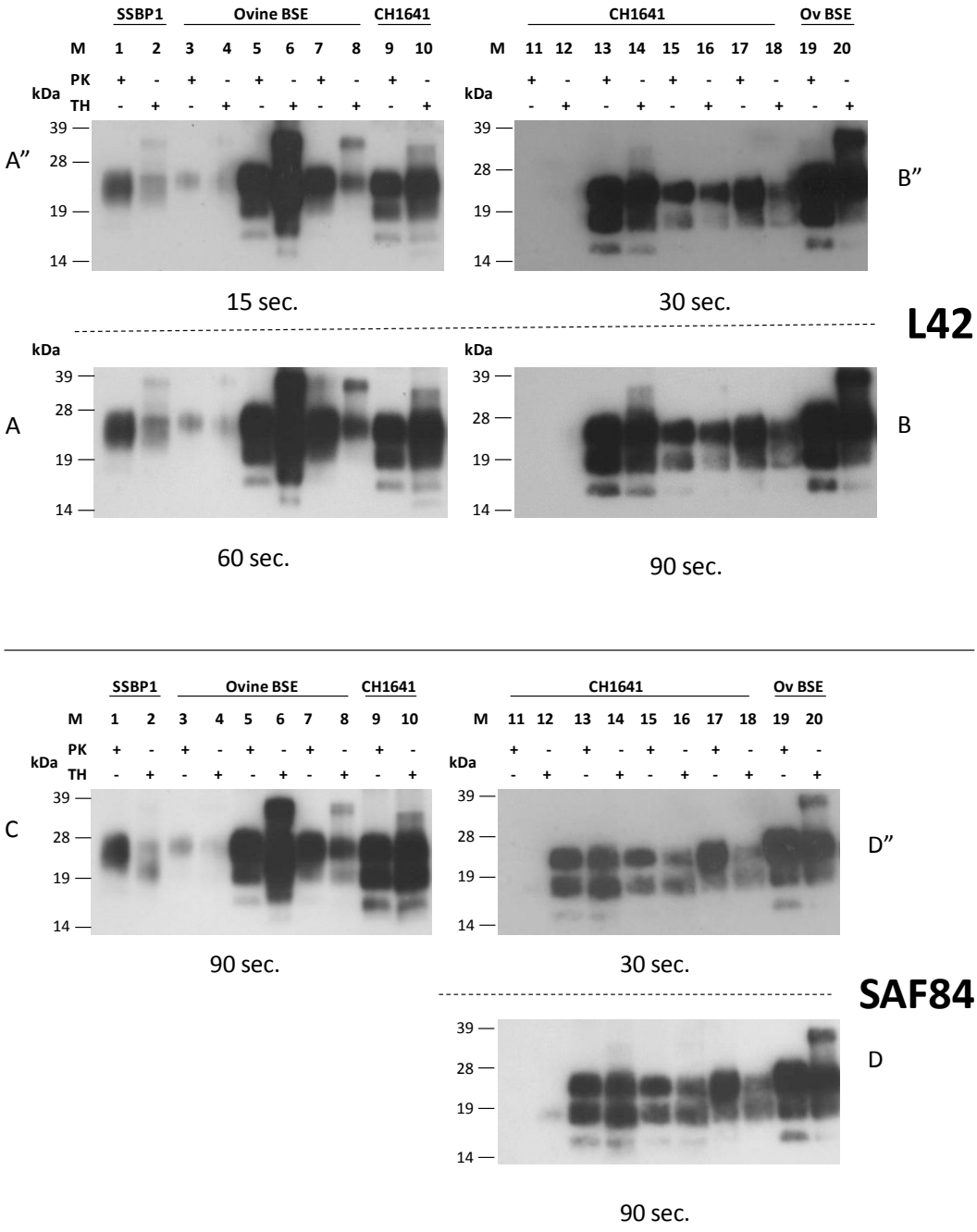


Figure 4.1: Western blot analysis of panels of ovine strains digested with either PK 100 µg/ml for 1 hour at 37ºC or TH at 150 µg/ml for 1 hour at 70ºC (as indicated; +/-). 3.3 µl of each 10% (w/v) cerebellum homogenate sample was loaded per lane and molecular mass markers are indicated in kDa; Lanes 1 & 2: (SSBP1) VRQ/VRQ, Lane 3 & 4: Ov BSE (1693/03), Lanes 5, 6, 19 & 20: Ov BSE (0392/04), Lanes 7 & 8: Ov BSE (0654/04), Lanes 9, 10, 13 & 14: CH1641 (J2935), Lanes 11 & 12: CH 1641 (J2916), Lanes 15 & 16: CH1641 (J3011), Lanes 17 & 18: CH1641 (VLA1). Membranes A”, A, B” & B were probed with mAb L42, membranes C, D” & D were probed with mAb SAF84. Western blot exposures were taken at different time points; 15, 30, 60 and 90 seconds.

Recently, using multiplex immunoblot approach (triplex-WB), for WB detection with L42 and SAF84 antibodies, CH1641 displayed a characteristic dominant diglycosylated PK PrP^{res} band after binding with L42, and with SAF84 having roughly equal intensities between di- and monoglycosylated PrP^{res} (Jacobs *et al.* 2011). Therefore, we compared the different glycoprofiles obtained for PK PrP^{res} of CH1641 and ovine BSE samples after binding with mAbs L42 and SAF84 on the same blot at different exposure time. As a result, CH1641 (J3011) isolate revealed a PrP^{res} with a glycoprofile of higher intensity for the diglycosylated band after probing with L42 (Panel B" & B, lanes 15 & 16 respectively). In contrast, it was difficult to detect variation in the intensities between the di- and monoglycosylated bands with other CH1641 samples detected by both antibodies and whatever the condition of digestion.

***Samples probed with the mAb P4* (*epitope 93-WGQGGSH-99*):** Generally, there was a clear variation in the amount of P4-binding-PrP^{res} produced between individual samples for each strain. The SSBP1 sample and the BSE samples were distinguished by TH digestion as previously reported (Owen *et al*., 2007). Both TH digested SSBP1 (Figure 4.2: Panel A, lane 2) and CH1641 (J2935) (Figure 4.2: Panel A, lane 10) isolates showed closely similar molecular profiles with apparent molecular mass of 36, 28, 23 kDa together with a lower band of ~ 15-17 kDa. This molecular profile was consistent in another Western blot analysis (Figure 4.2: Panel C, lanes 1 and 2).

Two ovine BSE samples (0392/04) (Figure 4.2: lanes 6 & 20) and (0654/04) (Figure 4.2: lane 8) had a different molecular weight to CH1641 (J2935); between 38 and 17 kDa, and 38 and 23 kDa banding patterns for TH PrP^{res}, respectively. Both Ov BSE (1693/03) (Figure 4.2: lanes 3 & 4) and CH1641 (J2916) (Figure 4.2: Panel B, lane 11 & 12) hardly showed any detectable PrP^{res} bands. The other two CH1641 (J3011 and VLA1), showed faint signals of approximately 26 kDa PK PrP^{res} (Figure 4.2: lanes 15 & 17) and 38 and 27 kDa of TH PrP^{res} (Figure 4.2: panels B, lanes 16 and 18). Increasing the amount of brain homogenate analysed (either directly or following NaPTA precipitation) did not enhance the detection signals of ovine BSE (1693/03), CH1641 (J2916), CH1641 (J3011), and CH1641 (VLA1) (data not shown). Therefore, the observed low P4 reactivity generated by TH full length PrP^{res} confirm the low level of full length PrP^{res} in CH1641 (J3011) and (VLA1) samples as showed by L42 and SAF84 mAb detection producing much higher signals (Figure 4.1). Overall, both experimental ovine BSE and CH1641 isolates demonstrated

significant heterogeneity in the levels of PrP^{res} present in the cerebellum during clinical disease. In addition, for TH resistant/ P4 reactive PrP^{res} fragments, BSE isolates gave consistent banding patterns whereas CH1641 isolates produced two distinct banding patterns with CH1641 (J2935) being distinct from the CH1641 isolates (J3011) and (VLA1).

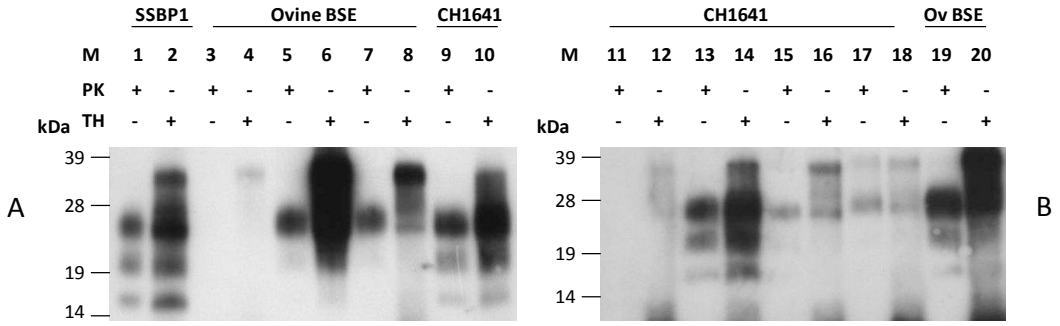


Figure 4.2 (Panel A and B): Western blot analysis of panels of ovine strains digested with either PK 100 µg/ml for 1 hour at 37ºC or TH at 150 µg/ml for 1 hour at 70ºC (as indicated; +/-). 3.3 µl of each 10% (w/v) cerebellum homogenate sample was loaded per lane and molecular mass markers are indicated in kDa; Lanes 1 & 2: (SSBP1), Lane 3 & 4: Ov BSE (1693/03), Lanes 5, 6, 19 & 20: Ov BSE (0392/04), Lanes 7 & 8: Ov BSE (0654/04), Lanes 9, 10, 13 & 14: CH1641 (J2935), Lanes 11 & 12: CH 1641 (J2916), Lanes 15 & 16: CH1641 (J3011), Lanes 17 & 18: CH1641 (VLA1). Membranes A & B were probed with mAb P4.

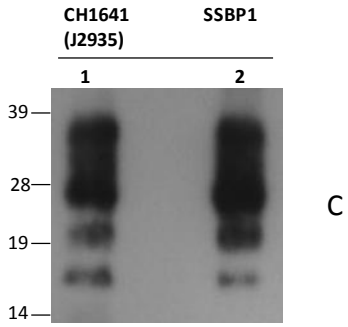


Figure 4.2 (Panel C): Western blot analysis of CH1641 (J2935) and SSBP1ovine strains digested with TH at 150 µg/ml for 1 hour at 70ºC. 3.3 µl of each 10% (w/v) cerebellum homogenate sample was loaded per lane and molecular mass markers are indicated in kDa. Lane 1: CH1641 (J2935), Lane 2: SSBP1. The membrane was probed with mAb P4.

4.2.2. Detection of PrP fragment CTF14:

Previous reports using Western blotting to discriminate between CH1641 and ovine BSE, showed that digesting "CH1641-like" scrapie with TH was characterized by the unique accumulation of a more C-terminally cleaved PrP fragment of 14 kDa, called (CTF14), when using SAF84 for PrP^{res} detection (Nicot and Baron, 2010, Baron *et al*., 2008). This fragment was also detected in the absence of any protease digestion. Therefore, I applied the same reported method to my ovine samples in order to establish whether they could be differentiated through the detection of additional C-terminal PrP^{res} cleavage fragments (i.e. CTF14). Samples of SSBP1, ovine BSE (0654/04) & CH1641 (J2935) isolates were digested with 1 mg/ml TH for a total of 4 hours and probed with SAF84. With my ovine samples, neither the undigested samples nor samples digested with TH for 4 hours (Figure 4.3) or PK for one hour (data not shown), have produced C-terminally cleaved unglycosylated PrP fragment of 14 kDa that are reported to characterise CH1641 and CH1641- like scrapie.

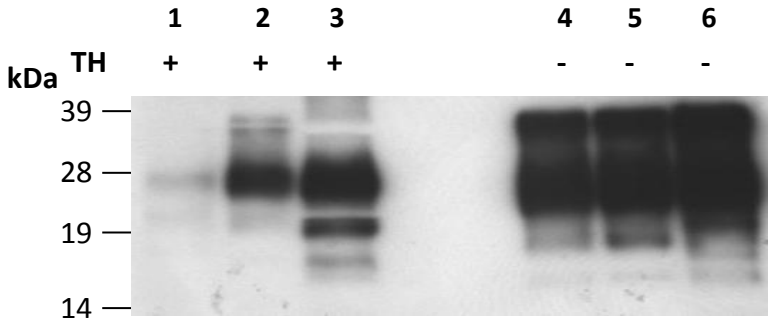


Figure 4.3: Western blot analysis of 10% (w/v) TSE affected cerebellum isolates for the detection of CTF 14 fragments. Lanes 1-3 represent 5 µl of digested samples of SSBP1, ovine BSE (0654/04) & CH1641 (J2935) respectively. These samples were digested with 1 mg/ml TH for a total of 4 hours. Lanes 4-6 represent 5 µl of each original sample (diluted 1: 3 in PBS) without protease treatment. PrP was detected with mAb SAF84.

4.2.3. Differential protease susceptibility of ovine prion isolates:

By qualitative comparing of signal intensities of PK PrP^{res} and TH PrP^{res} of TSE affected samples included in this study (Table 4.1), I was able to detect differences in the amount of PrP^{res} produced as a result of digesting particular samples with either PK 100 µg/ml for 1 hour at 37ºC or TH at 150 µg/ml for 1 hour at 70ºC. These amounts of proteases were optimised to the minimum amount required to remove all PrP^{C} (data not shown). For SSBP1, Ov BSE (1693/03), Ov BSE (0654/04) and CH1641 (VLA1) isolates and detecting PrP^{res} using core antibodies (Figure 4.1, Panel A to D, lanes 1 and 2, lane 3 and 4, lanes 7 and 8, and lanes 17 & 18), although each lane was loaded with 3.3 µl of digested materials, TH PrP^{res} was at lower levels than PK PrP^{res}. Approximately, similar levels of PK PrP^{res} and TH PrP^{res} were detected for other samples (Figure 4.1: Ov BSE 0392/04: lanes 5, 6, 19 & 20, CH1641 (J2935): lanes 9, 10, 13 & 14 and CH1641 (J3011) lanes 15 & 16). Equivalent results were produced with antibodies SAF84 and L42.

I then looked for possible quantitative differences of protease-resistant PrP^{Sc} produced by a range of classical scrapie isolates and different regions of brain tissues from these scrapie affected animals (Figure 4.4). The relative levels of TH and PK resistant PrP^{Sc} for between scrapie affected animals was not consistent with some displaying very similar levels of TH PrP^{res} and PK PrP^{res} species (0678/03, 1776/02, 0575, 0455/03, 0615/03 and 0635/03), whilst others displayed PrP^{Sc} with greater susceptibility to TH digestion than to PK (1275/02 and 1276/02). This was consistent when analysing protease-resistant PrP^{Sc} from different regions of the same brain for these animals. Furthermore, these experiments also revealed that in certain animals there was clear variation in differential protease susceptibility between different brain regions (0226/03 and 0284/97). In addition, certain samples produced additional C-terminal PrP^{res} cleavage fragments. This was demonstrated in detecting faint PK PrP^{res} fragments of ~ 15 kDa from three classical scrapie affected sheep: caudal medulla of animals 1776/02 and 0575/00 (Figure 4.4, panel B, lanes 7 & 9 respectively), and cerebellum samples of 1275/02 and 1276/02 affected sheep (Figure 4.3, panel E and F, lanes 1 respectively). Since these fragments were not detected from tissue treated with TH, these fragments seem to result from PK cleavage of full length PrP^{Sc} rather than endogenous proteolytic process.

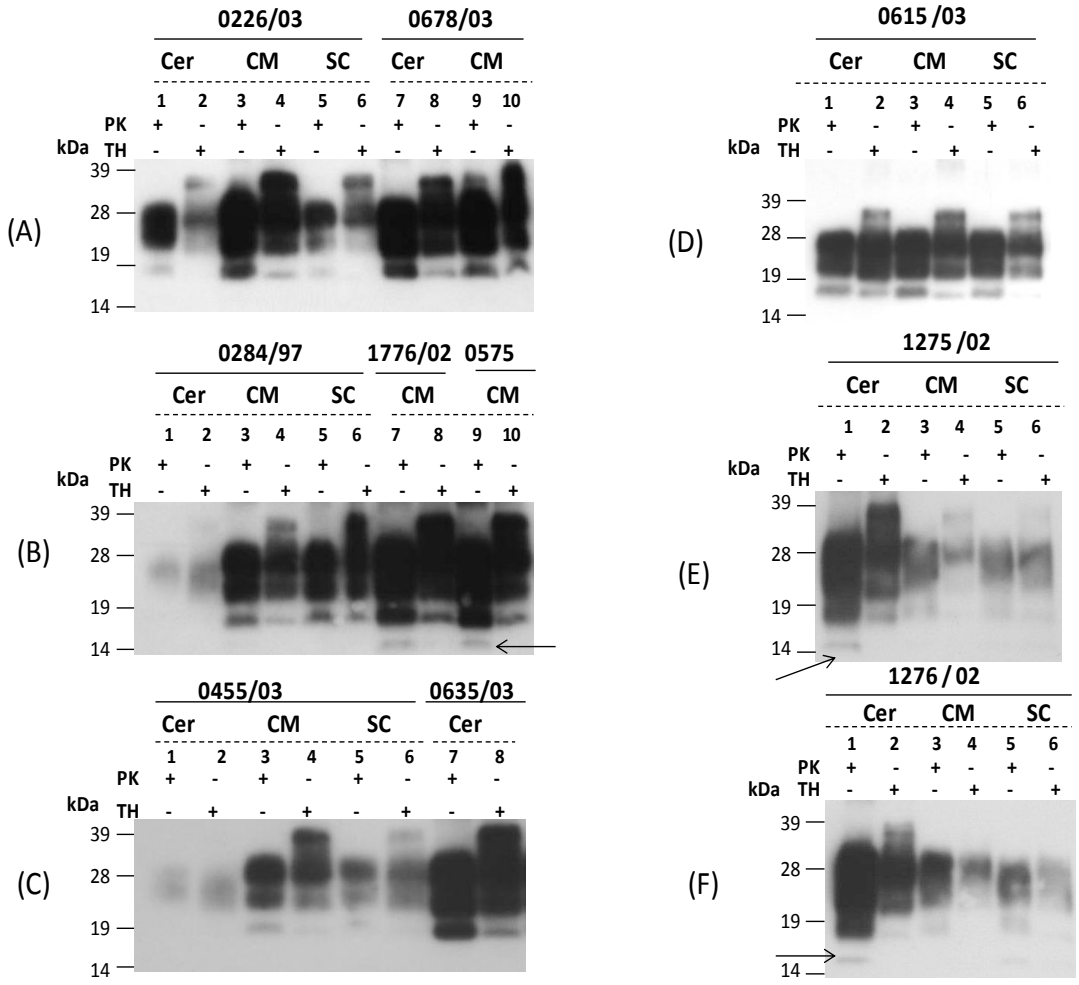


Figure 4.4: Western blot analysis of panels of different brain regions from classical scrapie affected sheep digested with either PK 100 µg/ml for 1 hour at 37ºC or TH at 150 µg/ml for 1 hour at 70ºC (as indicated; +/-). 3.3 µl of each 10% (w/v) brain region homogenate sample was loaded per lane; Cerebellum (Cer), Caudal Medulla (CM) and Spinal Cord (SC) samples were analysed. Membranes were probed with mAb SAF84. Molecular mass markers are indicated in kDa. C-terminal PrP^{res} cleavage fragments are indicated by arrows.

4.3. Discussion:

Multiple prion strains have been isolated from sheep after characterisation by their relative incubation periods and pattern of brain vacuolation in different inbred lines of mice (Bruce, 2003, Bruce and Fraser, 1991, Dickinson, 1976). In sheep, there are mainly four TSE types that have been recognized: classical scrapie, CH1641 scrapie, experimental BSE and atypical scrapie Nor98 (Benestad *et al*., 2003, Stack *et al*., 2002, Hope *et al*., 1999). In addition, classical scrapie passaged in mouse bioassays revealed the description of approximately 20 distinct mouse-adapted ovine prion strains, based on patterns of brain vacuolation and length of incubation times (Bruce, 2003, Bruce *et al*., 2002). Different typing methods have been used to differentiate between ovine TSE strains; including mouse bioassays, IHC or biochemical tests like Western blotting using PrP specific antibodies.

With regard to rapid assays, using conventional Western blotting methods, atypical scrapie had multiple PK PrP^{res} band patterns and a characteristic lower band of around 11 kDa which is different from the banding pattern of other animal prion diseases. Discrimination between PK PrP^{res} in classical scrapie and ovine BSE can be achieved and is based on: (a) the high concentration of the diglycosylated fraction in ovine BSE compared to that in scrapie infected samples (Thuring *et al*., 2004, Stack *et al*., 2002). (b) The molecular mass of the nonglycosylated band which is ~1.6 kDa smaller in sheep BSE than in sheep scrapie. However, it had been noted that the use of glycoform ratios to distinguish ovine BSE and scrapie did not produce consistent findings and showed different observations between laboratories (Baron *et al*., 2000, Baron *et al*., 1999, Hill *et al*., 1998). The distinct molecular weight of the nonglycosylated PrP^{res} has been further developed to describe the differential availability of antibody epitope binding sites after PK digestion; relative levels of binding of antibodies such as 66.94b4, 6H4, R521 in comparison to P4, with low reactivity of P4 with PrP^{res} in sheep BSE. This discriminatory method was based on differences in the location of the N-terminal cleavage site for PK PrP^{res} between BSE and scrapie and was considered a robust method for routine diagnosis of ovine BSE (Stack *et al*., 2006, Thuring *et al*., 2004).

With all diagnostic methods mentioned for strain typing, there is still a need for a diagnostic method that could confirm the presence of BSE in small ruminants

particularly after the detection of BSE in two cases of goat in Europe (Spiropoulos *et al*., 2011, Jeffrey *et al*., 2006b, Eloit *et al*., 2005). Western blotting has been applied and recommended to diagnose BSE in mice and small ruminants (Spiropoulos *et al*., 2011, Thuring *et al*., 2004, Stack *et al*., 2002). This method is cost effective and results can be obtained within short timescales which is suitable for massive surveillance.

Although, BSE has not been isolated from ovine field cases, it might be present and there is more need to accurately differentiate BSE from strain(s) such as CH1641. The unusual CH1641 isolate has molecular similarities to experimental ovine BSE. Although it is a rare form of ovine TSE, CH1641 and CH1641-like isolates have been isolated from natural cases from a number of European countries, and propagated experimentally in both sheep and transgenic mice (Baron and Biacabe 2007, Foster *et al*., 1988).

In a previous study, using TH for digesting TSE affected brain tissue produced PrP^{res} on Western blots that successfully distinguished ovine BSE from classical scrapie (Owen *et al*., 2007). Furthermore, TH produced a nonglycosylated PrP fragment of 14 kDa in CH1641 and in "CH1641-like" natural scrapie isolates (Nicot and Baron, 2010). Therefore, I extended previous work to the identification of PrP^{res} in the cerebellum of scrapie affected sheep using two proteases PK and TH and the analysis of the resulting fragments by Western blotting.

When detecting protease-resistant fragments with the P4 antibody, my results demonstrate that one CH1641 (J2935, ARQ/AHQ), showed features of having banding patterns indistinguishable to that shown by a classical scrapie strain SSBP1. It is worth noting that CH1641 (J2935) showed molecular weights similar to classical scrapie in three analyses and this was different from other CH1641 isolates. In contrast, other CH1641 isolates (J3011 and VLA1) both have AHQ/AHQ *Prnp* genotypes and showed molecular profiles that were similar to Ov BSE (0654/04, ARQ/ARQ and 0392/04, ARQ/ARQ).

Ov BSE (1693/03, ARQ/ARQ) and CH1641 (J2916, ARQ/AHQ) did not give a measurable level of PrP^{res} clearly due to low levels of PrP^{res} in both samples demonstrating that the level of PrP^{res} in a defined brain region during clinical disease is highly variable for individual animals.

Although CH1641 (J2935) together with CH1641 (J3011) and (VLA1) samples, showed readily detectable levels of PrP^{res} when digested with PK & TH and probed

with mAbs L42 & SAF84, there were variations in the PrP^{res} detected when probed with mAbs P4. mAb P4 had high level binding to CH1641 (J2935) but showed low binding to CH1641 (J3011), and (VLA1) samples. This reflects the reduced availability of P4 epitope in the latter two samples when digested with TH as a result of low levels of full length PrP^{res} possibly resulting from the effect of endogenous proteases to the full length PrP^{Sc} contents in these samples or due to the *in vitro* cleavage by TH at unfavourable sites in the N-terminus of PrP^{Sc}. IHC investigation of brains of sheep infected experimentally with BSE or CH1641 support these findings, as data showed marked increase in the detection of intraneuronal and intraglial labelling with anti C terminal antibody of the PrP molecule in comparison to detection with mAb P4 (Martin *et al*., 2005, Jeffrey *et al*., 2003, Jeffrey *et al*., 2001). Furthermore, IHC studies revealed that CH1641 showed a distinctive PrP^{d} profile and a cleavage site more towards the C terminus compared to PrP^{d} from BSE experimentally infected sheep (Jeffrey *et al*., 2006b, Gonzalez *et al*., 2005). These variations in antibody labelling patterns might relate to variation in the truncation sites of different strains of PrP^{d} as a result of variations in enzymatic activities or other lysosomal biochemical reactions (Martin *et al.* 2005). However, the relatively high level of P4 binding to CH1641 (J2935) reported here, may indicate considerable variation in the endogenous protease cleavage of the total pool of PrP^{res} in CH1641 infected cerebellum samples.

Although the relative binding of sheep scrapie to P4 in comparison to BSE was one of the main features in differentiation between typical scrapie and experimental ovine BSE (Stack *et al*., 2006), two of my ovine BSE samples (0392/04 and 0654/04) showed detectable level of PrP^{res} after PK digestion and detection with P4, but this was less than the detection level with L42 and SAF84 and without clear banding patterns.

A new technique using multiplexed-antibody detection and comparison of mAbs L42 and SAF84 binding levels has been shown to distinguish BSE from CH1641. Both ovine experimental BSE and CH1641 demonstrated strong binding to both L42 and SAF84 mAb after PK digestion, but CH1641 produced diglycosylated PK PrP^{res} that was the dominant band with L42 detection but gave equal intensities between di- and monoglycosylated bands with SAF84 detection (Jacobs *et al*., 2011). This was reported to form a strain typing assay. Within my samples, the glycoform ratios were

indistinguishable between L42 and SAF84 for other CH1641 and ovine BSE samples. However, given the low number of isolates examined, their descriptions need verifying with further samples.

Variations in the conformational stability and solubility (CSSA) in GdnHCl between CH1641 and BSE have also been recorded (Pirisinu *et al*., 2011). This assay was based on the concentration of GdnHCl able to solubilise 50% of PrP^{Sc} ($GdnHCl_{½}$ values). This study reflected the higher resistance of ovine BSE PrP^{res} to solubilisation with $GdnHCl_{½}$ values > 3.8 mol/L. In contrast, CH1641 and scrapie showed $GdnHCl_{½}$ values of 2.0 - 2.8 mol/L and 2.2 mol/L. These demonstrated conformational stabilities reflect the divergent physicochemical properties of PrP^{res} between strains.

Studies of the molecular traits of CH1641-like scrapie in transgenic mice have resulted in the description of C-terminal PrP^{res} cleavage fragments (Baron *et al*., 2008). Furthermore, this endogenous, more C-terminally cleaved, PrP fragment of 14 kDa in its nonglycosylated form, was a feature in CH1641, in "CH1641-like" natural scrapie isolates and in the 87V scrapie strain and was absent from BSE samples (Nicot and Baron, 2010). Moreover, recent studies demonstrated the clear discrimination between CH1641-like natural isolates in sheep and goat from BSE based on the presence of C-terminally cleaved, PrP fragment of 14 kDa detected by SAF84 (Vulin *et al*., 2011). However, in the present study these PrP fragments of this molecular mass were not detected in CH1641 samples with SAF84. This might be due to my samples being obtained from experimentally infected sheep. The PrP^{Sc} inoculua have been passaged several times in mice or sheep which might affect the molecular features after treatment with proteases. Also, the results presented here were obtained after digesting samples with 100 µg/ml PK, which is different from the methodology of Baron *et al*., 2008. In my study, samples were also digested with 1 mg/ml TH that is similar to the methodology of Nicot and Baron, 2010, therefore, it is not apparent that variations in the methodologies of analysis contribute to the differences in the results. Subtle methodological differences, geographical origins of samples, and sample preparations have been found to cause variations in the glycotypes and some diversity in the molecular masses of fragments (Gonzalez *et al*., 2010, Sweeney *et al*., 2000).

Interestingly, a C-terminal PrP^{res} cleavage fragment of ~ 15 kDa was detected with some classical scrapie affected field cases. This fragment was not seen in all animals and was not consistently seen across multiple brain regions within an individual sheep. This data suggests that PrP^{Sc} can have different physicochemical properties towards proteases and there are individual variations between animals and also PrP^{Sc} produced by different brain regions within the same animal.

Since SSBP1, CH1641 and ovine BSE were all cerebellum tissues which is not the optimum recommended sample for detection of classical scrapie and BSE (OIE Terrestrial manual, 2009), it might explain why PrP^{res} was not readily detected from certain samples (CH 1641, J2916) and (ovine BSE, 1693/03). Therefore, I examined the impact of the anatomical sources of brain tissue used on the detection of PrP^{res} using both PK and TH from 10 different classical scrapie affected animals. Cerebellum regions of 1275/02 and 1276/02 scrapie affected sheep contained more PrP^{res} than caudal medulla and spinal cord of each animal. However, it was hypothesised previously that different cell types process abnormal PrP in different ways (Gonzalez *et al*., 2003), this might explain how different brain tissues accumulate PrP^{Sc} to different extents. Furthermore, Dron *et al*., (2010) suggested that certain nerve cells might be more liable to endogenous proteolysis than others. Such tissue-specific differences may offer different environments for PrP^{Sc} selection within a single host and therefore result in dominant PrP^{Sc} conformers with different susceptibilities to exogenous proteases (Collinge and Clarke, 2007). This was confirmed by Stack et al, 2006, when they reported brain region specific differences in the molecular profile of ovine PrP^{res}. Their study demonstrated by Western blot that PK PrP^{res} from the same animal looked like experimental BSE in sheep after sampling of the whole brain stem, and gave the usual scrapie profile when caudal medulla was sampled. However, on the basis of the discriminatory IHC results, the sample gave a scrapie pattern. Therefore, biochemical typing results should be interpreted with caution until other tests such as IHC and bioassay data confirm the results. A further report concluded that endogenous proteolytic digestion that might occur before the *in vitro* analysis of samples may affect trimming of full length PrP^{Sc} and in turn the interpretation of the molecular profiles of such strains (Dron *et al*., 2010).

In my assay conditions, all samples were represented by 3.3 µl of protease treated brain tissue, and it was noted that there was variation in the PrP^{res} signal intensity produced for individual samples, dictated by treatment with either PK or TH. The present data showed that some samples contained more PK PrP^{res} than TH PrP^{res} whilst other contained similar levels of these two resistant isoforms. One molecular trait was observed consistently with some animals across neuroanatomical regions whilst other animals displayed both traits. The level of differential protease resistance did not appear to be influenced by genotype or PrP^{Sc} strain. Therefore, these findings suggest that protease-resistant PrP isoforms vary between individual animals/ brain regions which might be related to unidentified host factors.

The availability of PK-sensitive isoforms of PrP^{Sc} has been well documented for rodent scrapie models and in human CJD (Pastrana *et al*., 2006, Tzaban *et al*., 2002, Safar *et al*., 1998). My result demonstrates that for ovine prion strains TH-sensitive PrP isoforms exist for numerous isolates and their presence varies between individual sheep and brain regions.

Therefore, in view of my analysis of classical scrapie, ovine BSE and CH1641 isolates, I conclude that differences in the protease resistant PrP^{Sc} molecular profiles revealed with multiple antibodies can be apparent between individual isolates of the same TSE strains and reflect the biological diversity of PrP^{res}. Protease-resistant PrP for individual isolates for CH1641, ovine BSE and classical scrapie had divergent physicochemical properties towards proteases, and this was independent of *Prnp* genotype and TSE strain in this study. However, the genotype variability among my samples was low and the analysis of further samples could be required to fully define any effect of the genotype or breed on the results.

Previous reports suggested that biochemical methods for scrapie strain discrimination did not show clear evidence supporting the effect of genotype on the molecular profile of scrapie strains (Nonno *et al*., 2003, Sweeney *et al*., 2000). In addition, a recent publication showed that *Prnp* polymorphisms did not account for PrP^{d} profile variation and pathological phenotype diversity (Gonzalez *et al*., 2010). In contrast, a strong association was seen between differences in PrP^{Sc} patterns and vacuolation profiles of natural scrapie cases and PrP genotype using IHC methodology (Spiropoulos *et al*., 2007). It is possible that *Prnp* genotypes can select for certain strains under field conditions and the strains dictate pathological features. This could

account for the different conclusions across these studies. However, my presented data clearly shows that for defined strains under experimental conditions that the PrP^{Sc} accumulation and molecular phenotype of a particular TSE strain can vary between individuals. This conclusion was also demonstrated in a previous experimental study (Yokoyama *et al*., 2010), where two sheep of identical genotype, but different breeds, responded differently to inoculation with a single scrapie strain. These differences were observed in the incubation period of disease, molecular phenotype of propagated PrP^{Sc} conformers by Western blot analysis, and pathological lesions in transgenic mice. This in turn indicates that prion propagation could be affected by other factors and there is selection for certain PrP^{res} conformers by individuals during intra- and interspecies transmission (Ushiki-kaku *et al*., 2010, Yokoyama *et al*., 2010).

In conclusion, my study highlights the availability of TH-sensitive, PK PrP^{res} isoforms in sheep scrapie and the biological diversity of PrP^{res} of different prion strains. Significantly I have shown that the detection of particular isoforms of PrP^{res}, which depend on the stability of PrP^{Sc} conformers towards exogenous proteases to yield distinct protein fragments, is variable even between individual sheep experimentally infected with the same strain, and between distinct brain regions for naturally infected animals. This was highlighted for CH1641 where 2 distinct molecular phenotypes were noted, further studies of field cases of CH1641-like samples are needed to define these molecular profiles further.

Chapter 5. *In Vitro* Amplification of Ovine Prion Strains in Substrates of Different *Prnp* Genotypes

5.1. Introduction:

Since the proposition of the "protein only hypothesis" (Prusiner 1998 & 1982), major research efforts have investigated the epidemiology, pathogenesis, diagnosis and therapeutic treatment or vaccination against prion diseases. The development of *in vitro* prion propagation systems to help understand the molecular mechanisms of such diseases, have been difficult subjects to tackle in the neuroscience research field (Ryou and Mays, 2008). The past decade has seen significant developments in the molecular diagnostic techniques of prion diseases, but as yet, we are still limited to post-mortem examination (Ryou and Mays, 2008). However, continuous advancements in the *in vitro* conversion assays offers a way to understand the mechanism of PrP^{Sc} propagation and a method to improve early non- invasive diagnosis of TSE diseases particularly in pre-clinical cases. The developments of PMCA and QUIC technologies have shown great potential for the development of an ultra- sensitive diagnostic test through amplification of prion *in vitro*. The present study looks to further apply PMCA to the diagnosis of sheep prion diseases.

The PMCA technique was developed by Soto and colleagues (Saborio *et al*. 2001), and has been further modified to a technique called sPMCA (Castilla *et al*., 2005a). sPMCA has enabled high level amplification and sensitive detection of PrP^{Sc} in different biological materials from scrapie infected rodents in the clinical and preclinical stages of disease (Gonzalez-Romero *et al*., 2008, Maluquer de Motes et *al*., 2008, Murayama *et al*., 2007, Saa *et al*., 2006, Castilla *et al*., 2005, Soto et *al*., 2005). It has also been applied to detect ovine scrapie PrP^{Sc} in blood (Thorne and Terry, 2008), milk (Gough *et al*., 2009, Maddison *et al*., 2009), as well as saliva (Gough *et al*., 2012, Maddison *et al*., 2010) and faeces (Terry *et al*., 2011), and within environmental samples (Gough and Maddison 2010, Maddison *et al*., 2010 a & b). In addition, the technique also demonstrated successful amplification of PrP^{Sc} from a range of mammalian species including CWD in deer (Angers *et al*., 2009, Haley *et al*., 2009, Kurt *et al*., 2007), BSE in cattle (Soto *et al*., 2005) and vCJD in humans (Jones *et al*., 2009 & 2007). In some of these studies, experimental animal infection together with the PMCA technique were applied to improve the understanding of PrP^{Sc} dissemination and routes of disease transmission. Other studies have looked at the ability of PMCA to model the genetic susceptibility of sheep to scrapie (Bucalossi *et al*., 2011). In addition, PMCA has been used in

comparative studies to define the specific infectivity generated in PMCA compared to that of brain derived in vivo infectivity (Klingeborn *et al*., 2011).

In this report, I investigated the potential sPMCA amplification of different ovine prion strains within distinct ruminant substrates: ovine substrates of distinct *Prnp* genotypes as well as bovine substrate. Statistical analysis was also performed with binomial logistic regression test using SPSS software for statistical computing. The aim was to use sPMCA to investigate the complex interaction of *in vitro* specific naturally occurring ruminant PrP sequences and prion strain types during prion propagation.

5.2. Results:

5.2.1. Determining the sensitivity of sPMCA for the detection of PrP^{Sc}:

To assess the sensitivity of the PMCA technique for the detection of animal TSE diseases, different dilution series of 10% infected brain tissues for bovine BSE, ovine BSE, scrapie of *Prnp* genotype VRQ/VRQ and ARQ/ARQ were spiked into prion-free ruminant brain substrates (Table 5.1). Four PMCA rounds were performed for each sample; a total of 192 cycles over 4 days. Further samples were prepared and amplified in exactly the same way but were non-spiked controls; no controls yielded any protease-resistant PrP^{Sc}. Samples were digested with PK and analysed by Western blot using antibody SHA31.

Data show the high efficiency amplification of bovine BSE PrP^{Sc} in healthy bovine brain substrate. The de novo formed PrP^{Sc} were detected following amplification of 100 ng of the BSE brain spike (Table 5.1 and Figure 5.1: A). Similarly, high amplification efficiency was achieved with bovine BSE infected brain tissue spiked into healthy homozygous ARQ ovine substrate, detecting just 10 ng of brain (Table 5.1; Figure 5.1: B). Both healthy bovine substrate and homozygous ARQ Ovine substrate supported amplification of homozygous ARQ ovine BSE infected brain tissue, PrP^{Sc} from 1 and 0.1 ng of brain being detected respectively (Table 5.1; Figure 5.1: C & D). Furthermore, SSBP1 strain (VRQ/VRQ) showed only low levels of PrP^{Sc} being amplified but this was detected from just 0.1 ng of brain (Table 5.1; Figure 5.1: E). This amplification was only seen in VRQ/VRQ substrate. No

amplification was seen in ARQ, ARR or bovine substrate. Homozygous ARQ PrP^{Sc} spike demonstrated very low levels of PrP^{Sc} amplification, but again PrP^{Sc} could be detected after very high dilutions of the brain spike (100 ng of brain spike was detected) (Table 5.1; Figure 5.1: F). None of the spiked bovine and ovine PrP^{Sc} brain homogenates were amplified in homozygous ARR substrate (data not shown). In addition, neither ovine nor bovine BSE could be amplified in VRQ substrate. None of the spiked non sonicated samples (amplification efficiency control samples) were detected on Western blots (data not shown). No detectable spontaneous protease-resistant PrP formation was observed in all control brain homogenate substrates samples that were subjected to sPMCA (data not shown).

Table 5.1: Dilution series of bovine BSE, ovine BSE, scrapie SSBP1 (VRQ/VRQ), and scrapie (ARQ/ARQ) infected brain tissue spiked into different brain homogenate substrate.

PrP^{Sc} Sample	Genotype and ID of spike	Dilution series analysed (ng TSE brain)	sPMCA Substrate	Limit of detection (ng TSE brain)
Bovine BSE	03/0572	10,000 – 100	VRQ/VRQ	No amplification (>10,000)
Bovine BSE	03/0572	1000 – 0.1	Bovine	100
Bovine BSE	03/0572	10,000 – 10	ARQ/ARQ	10
Bovine BSE	03/0572	10,000– 10	ARR/ARR	No amplification (>10,000)
Ovine BSE	ARQ/ARQ 1693/2036	1000 – 10	VRQ/VRQ	No amplification (>1,000)
Ovine BSE	ARQ/ARQ 1693/2036	10 – 0.01	Bovine	1
Ovine BSE	ARQ/ARQ 1693/2036	10 – 0.01	ARQ/ARQ	0.1
Ovine BSE	ARQ/ARQ 1693/2036	10,000 – 0.1	ARR/ARR	No amplification (>10,000)
Scrapie	SSBP1 VRQ/VRQ	10 – 0.01	VRQ/VRQ	0.1
Scrapie	SSBP1 VRQ/VRQ	10,000 -1,000	Bovine	No amplification (>10,000)
Scrapie	SSBP1 VRQ/VRQ	10,000 – 10	ARQ/ARQ	No amplification (>10,000)
Scrapie	SSBP1 VRQ/VRQ	10,000 – 1,000	ARR/ARR	No amplification (>10,000)
Scrapie	ARQ/ARQ 0678/2129	10,000 – 0.1	ARQ/ARQ	100

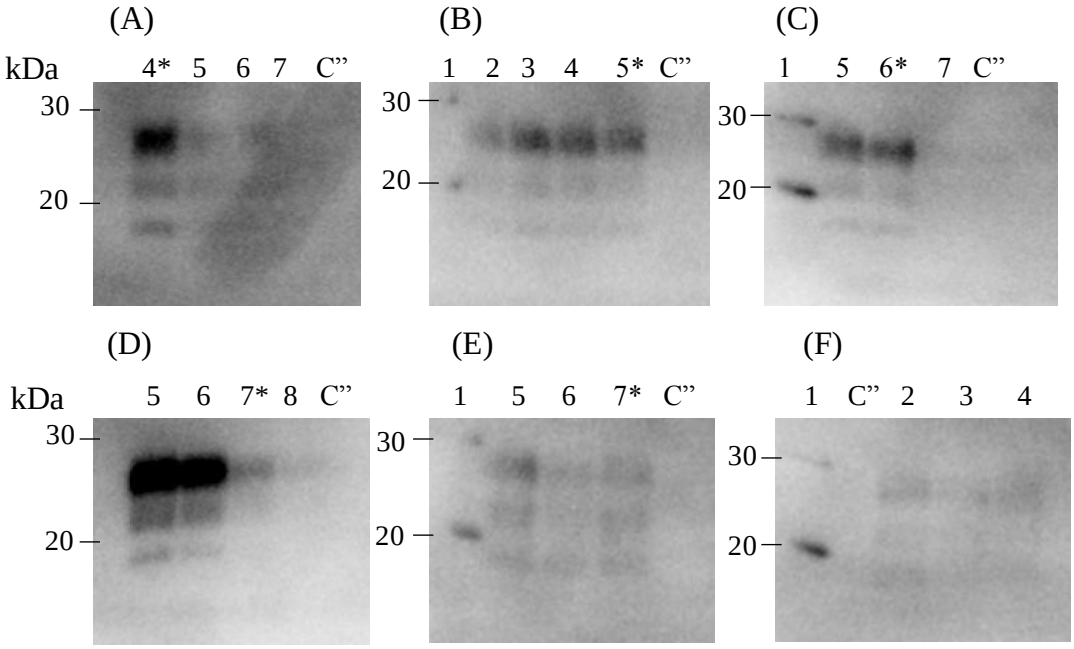


Figure 5.1: Serial PMCA analysis of ruminant prions: 10-fold dilution series (DS) of brain homogenate from clinically affected sheep with either scrapie or BSE, or cattle with clinical BSE were seeded into sPMCA reactions (lanes 2 to 8 represent 10000 to 0.01 ng of brain homogenate seed respectively). Each sample underwent four rounds of sPMCA and products were digested with PK before analysis of 5 µl of each sample on Western blots. PrP was detected with monoclonal antibodies SHA31 and P4 and molecular weight markers of 30 and 20 kDa are indicated. Control samples (C”) were brain homogenates from TSE-free bovine or ovine sources. All sPMCA reactions were carried out as described by Castilla *et al*., 2005. Bovine BSE was amplified and detected from 100 ng of brain homogenate seed spiked into healthy bovine (A), and 10 ng spiked into healthy ARQ/ARQ substrate (B). Ovine BSE was amplified and detected from 1 ng when spiked into bovine healthy substrate (C), and 0.1 ng spiked into ARQ/ARQ healthy substrate (D). Similarly, SSBP1 (VRQ/VRQ) was amplified and detected from 0.1 ng spiked into VRQ/VRQ healthy substrate (E). Homozygous ARQ PrP^{Sc} demonstrated very low amplification signals when spiked into ARQ/ARQ healthy substrate (F). (*) is the cut off point determined by Western blot for the highest dilution of brain homogenate seed from sheep with either scrapie or BSE, or cattle with clinical BSE amplified by PMCA.

5.2.2. Application of sPMCA to the amplification of sheep TSEs:

To assess the ability of the PMCA technique to amplify sheep scrapie, 50 or 100 µg of brain seed from a range of scrapie affected animals were amplified within PMCA brain homogenate substrates of *Prnp* genotypes; VRQ/VRQ, ARQ/ARQ and AHQ/AHQ . Up to five PMCA rounds for samples spiked with 50 µg TSE positive brain seed were carried out, and 10 rounds were performed for samples spiked with 100 µg of TSE positive brain homogenate. All samples were digested with 50 µg/ml PK and analysed by Western blotting detecting PrP^{Sc} with SHA31 mAb (Table 5.3).

5.2.3. Amplification of PrP^{Sc} spikes in VRQ/VRQ Substrate:

The VRQ/VRQ substrate supported amplification of the highest number of isolates. The detected PrP^{Sc} showed successful amplification over 10 rounds of classical scrapie from sheep with *Prnp* genotypes that have at least one VRQ allele using either 50 or 100 µg spike. Unexpectedly, after 5 rounds of amplification, two VRQ/VRQ spikes (0836/03 and SSBP1) were not detected in VRQ/VRQ substrate using 100 µg of spike although they were detected after 10 rounds, and after 5 rounds when using 50 µg spike. These variations in detection of such amplified PrP^{Sc} samples, after increasing the spike volume, might reflect variation in the efficiency of amplification between two different experiments. None of the PrP^{Sc} classical scrapie from the two homozygous ARQ animals (0678/03 and 0635/03) was amplified in VRQ/VRQ substrate, whatever the volume of spike. Similarly, CH1641 PrP^{Sc} (4 samples) was not amplified at round 5 or even during prolonged sPMCA over 10 rounds and increasing the volume of spike to 100 µg. In contrast, ovine BSE spikes demonstrated successful amplification at all rounds analysed except one isolate (1693/03) that was not detected at round one.

5.2.4. Amplification of PrP^{Sc} spikes in ARQ/ARQ Substrate:

Seeded ARQ/ARQ substrate reactions had different amplification efficiencies compared to VRQ/VRQ substrate. None of the classical scrapie or CH1641 samples amplified in ARQ/ARQ substrate using 50 µg spike after 5 rounds of sonication. However, after increasing the spike volume to 100 µg, 7 out of the 12 classical scrapie isolates were amplified to some extent. The two ARQ/ARQ classical PrP^{Sc} scrapie samples were detected either after 5 and 10 rounds (0678/03) or after 10

Table 5.3: PMCA Amplification of different PrP^{Sc} strains/isolates with distinct genotypes:

Strain	Isolate ID	Strain genotype	TSE-free substrate	VRQ				ARQ				AHQ			
			spike (µg of brain)	50	100			50	100			50	100		
			No. of rounds	5	1	5	10	5	1	5	10	5	1	5	10
Classical scrapie	1563/02	VRQ/VRQ		+	−	+	+	−	−	−	−	−	−	−	−
Classical scrapie	1276/02	VRQ/VRQ		+	−	+	+	−	−	−	−	−	−	−	−
Classical scrapie	1275/02	VRQ/VRQ		+	−	+	+	−	−	−	−	−	−	−	−
Classical scrapie	0923/03	VRQ/VRQ		+	−	+	+	−	+	+	−	−	−	−	−
Classical scrapie	0925/03	VRQ/VRQ		+	−	+	+	−	−	+	−	−	−	−	−
Classical scrapie	0836/03	VRQ/VRQ		+	−	−	+	−	−	−	−	−	−	−	−
Classical scrapie – SSBP1	SSBP1	VRQ/VRQ		+	−	−	+	−	−	−	+	−	−	−	+
Classical scrapie	0226/03	ARQ/VRQ		+	+	+	+	−	−	−	−	−	−	−	−
Classical scrapie	0455/03	ARQ/VRQ		+	−	+	+	−	−	−	+	−	−	−	−
Classical scrapie	0615/03	ARQ/VRQ		+	+	+	+	−	+	−	−	−	−	−	−
Classical scrapie	0678/03	ARQ/ARQ		−	−	−	−	−	−	+	+	−	−	−	−
Classical scrapie	0635/03	ARQ/ARQ		−	−	−	−	−	−	−	+	−	−	−	−
CH1641	J2916	ARQ/AHQ		−	−	−	−	−	−	−	−	−	−	−	−
CH1641	J2935	ARQ/AHQ		−	−	−	−	−	+	+	+	−	−	−	+
CH1641	J3011	AHQ/AHQ		−	−	−	−	−	+	−	−	−	−	−	+
CH1641	VLA1	AHQ/AHQ		−	−	−	−	−	−	−	+	−	−	−	+
Ovine BSE	1693/03	ARQ/ARQ		+	−	+	+	+	+	+	+	+	+	+	+
Ovine BSE	0654/04	ARQ/ARQ		+	+	+	+	+	+	+	+	+	+	+	+
Ovine BSE	0392/04	ARQ/ARQ		+	+	+	+	+	+	+	+	+	+	+	+

- Positive = yellow and negative = blue

rounds of sonication (0635/03) when using 100 µg of seed. Furthermore, classical PrP^{Sc} spike 0455/03 (ARQ/VRQ), SSBP1 (VRQ/VRQ) and CH1641 (VLA1-AHQ/AHQ) were detected after 10 rounds of PMCA reaction. Interestingly, the three ovine BSE spikes showed successful amplification independent of the volume of spike or number of PMCA rounds. Classical scrapie 0923/03 (VRQ/VRQ), 0925/03 (VRQ/VRQ) and 0615/03 (ARQ/VRQ), and CH1641 J3011 (AHQ/AHQ) were detected transiently at round 1 and/or round 5 and then not seen in later rounds. CH1641 J2935 (ARQ/AHQ) was detected at all rounds analysed, but with a decrease in signal intensity between rounds 5 and 10 (Figure 5.2). The kinetics of PrP^{res} propagation after each round of sonication was analysed for 0923/03 (VRQ/VRQ) and CH1641 J2935 (ARQ/AHQ). The detected PrP^{res} on each round showed an increase in PMCA-PrP^{Sc} amplification up to round 3 for 0923/03 and round 4/5 for CH1641 J2935, after which the PrP^{res} levels decreased (Figure 5.2).

5.2.5. Amplification of PrP^{Sc} spikes in AHQ/AHQ Substrate:

None of the classical scrapie PrP^{Sc} spikes or CH1641 scrapie samples were detected after 5 rounds of amplification in AHQ/AHQ substrate seeded with either 50 µg or 100 µg of brain seed. After 10 rounds of sonication, and seeding the reaction with 100 µg spike, amplified PrP^{Sc} of SSBP1 (VRQ/VRQ) and CH1641 (3 out of four samples, J2935 ARQ/AHQ, J3001 AHQ/AHQ and VLA1 AHQ/ AHQ) were detected. Again, seeding PMCA reactions with 50 or 100 µg of each ovine BSE spike demonstrated successful amplification of the three isolates independent of the number of PMCA rounds.

Statistical analysis of the result of amplification (binomial data; positive or negative) and the influence of other elements (strain, PrP^{Sc} genotype, substrate genotype, amount of seed, number of rounds) included in PMCA reactions showed significant association to the effect of strain (degree of freedom (df) = 2, $P < 0.001$), seed genotype (df = 1, $P < 0.01$), substrate genotype (df = 1, $P < 0.001$), and number of rounds (df = 2, $P < 0.01$). Furthermore, within the model including all variables, comparison of amplification between different strains showed that ovine BSE amplification were highly significant (df = 1, $P < 0.001$) while CH1641 (df = 1, $P= 0.088$) was less significant in comparison to classical scrapie. However, there was

no statistical difference between CH1641 and ovine BSE amplification which seems to be due to the lack of other genotypes rather than ARQ/ARQ for Ov BSE.

Further trial using Chi-Square test showed a significant difference in amplification between the three strains ($P < 0.0001$). At odds to the observed results within the model, there was a significant difference in amplification between ovine BSE and CH1641 ($P < 0.0001$)

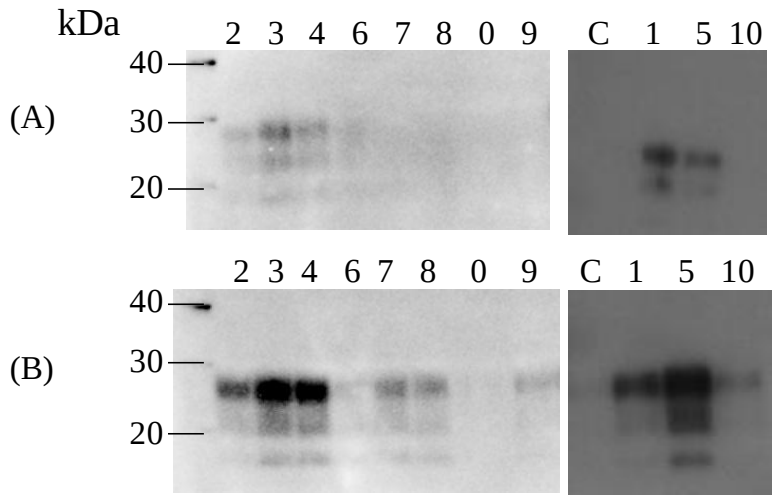


Figure 5.2: *In vitro* amplification of (A) classical scrapie VRQ/VRQ (0923/03) and (B) experimental ovine CH1641 ARQ/AHQ (J2935) over 10 rounds of sonication: 100 µg cerebellum homogenate of each strain was spiked into ARQ substrate to a final volume 100 µl. The mixture was subjected to 10 rounds of sPMCA reaction as described. After completing the reaction, 20 µl of each sonicated sample on each round was digested for 1 hour at 37ºC with PK (50 µg/ml). Typically, 5 µl of each digested sample was analyzed by Western blotting and probed with SHA31 antibody. Each lane is marked in the top with the number of rounds of amplification, 0 is spiked ARQ substrate which was not sonicated. "C" is ARQ substrate (control non-spiked sample subjected to sonication).

5.3. Discussion:

One of the main objectives of using the PMCA technique is to facilitate the rapid detection of PrP^{Sc} where the levels of PrP^{Sc} are undetectable by the current diagnostic methods (Thorne and Terry, 2008). Within the present study, different strains of ruminant prions were successfully amplified *in vitro* using sPMCA. Bovine BSE showed high conversion efficacy in both bovine brain homogenate substrate and ovine ARQ/ARQ brain homogenate substrate, and from a dilution of 100 ng and 10 ng brain seed. Also, SSBP1 classical scrapie strain demonstrated amplification in VRQ/VRQ substrate from a dilution equal to 0.1 ng scrapie positive brain material. Ovine BSE was also amplified in bovine or ovine ARQ substrate from as little as 0.1 ng of brain spike.

At present PMCA has only applied to a limited number of ovine TSE strains/ isolates. Here, PMCA reactions of 19 PrP^{Sc} isolates with different genotypes were examined to ascertain the possibility of prion amplification for the different combinations of prion seeds and *Prnp* genotypes substrates. This was illustrated in the successful amplification of 10 classical scrapie spikes (7 VRQ/VRQ and 3 VRQ/ARQ) out of 12 isolates in VRQ substrate with only ARQ/ARQ samples being refractory to amplification. AHQ substrate did not support such high efficiency amplifications with only a single classical scrapie sample amplified over 10 rounds. Ovine BSE was readily propagated in all substrates independent of the substrate genotypes while CH1641 was refractory to amplification in VRQ and AHQ substrates after 10 and 5 rounds respectively.

A preliminary trial (Priem *et al*., 2008) demonstrated the potential amplification of different bovine and ovine PrP^{Sc} isolates in distinct ovine and bovine PrP^{C} substrates. At the same time, previous studies showed that the success of PMCA and efficiency of amplification was influenced by prion strains and ovine PrP^{C} substrate genotype (Thorne and Terry, 2008), as well as by cross species compatibility between the PrP^{Sc} seed and PrP^{C} substrate (Soto *et al*., 2005). This is in agreement with my results where classical scrapie samples from sheep with *Prnp* genotype that have at least one VRQ allele were amplified successfully in VRQ/VRQ substrate.

In the present study, low levels or absence of amplification was seen when some classical VRQ/VRQ or VRQ/ARQ PrP^{Sc} were seeded into ARQ/ARQ substrate and when CH1641 was seeded into VRQ/VRQ substrate. These data highlight the impact of genotype on a template-substrate interaction for classical scrapie amplification *in vitro* (Panza *et al*., 2008, Kupfer *et al*., 2007, Bossers *et al*., 2000, Bossers *et al*., 1997). The efficiency of replication is clearly influenced by high homology between the amino acid sequences of a fibrillar template and a precursor substrate (Makarava *et al*., 2009). Therefore, mismatch between a template and a substrate might act as a barrier for amplification *in vitro*, and host susceptibility to TSE agent *in vivo* (Cancellotti *et al*., 2007). They concluded that as well as primary amino acid sequence homology between substrate and seed, the conformational similarity between the two components affect replication efficiency. For rodent scrapie strains, Atarashi *et al*., 2006, showed that two prion strains with the same PrP sequence but different conformations have differing abilities to convert the same mutated PrP^{C} in a cell culture. This conclusion is in agreement with the sPMCA data where seeding ARQ/ARQ healthy substrate with homozygous scrapie ARQ PrP^{Sc} required higher levels of spike or further rounds of sPMCA to amplify successfully compared to seeding with the three ARQ ovine BSE spikes.

In addition, low efficacy amplification might also be related to low levels of PrP^{Sc} molecules in the spiked dose and duration of amplification which was clearly demonstrated by using 50 or 100 µg of seed and detection of amplified products at different rounds of sonication for classical scrapie and CH1641 samples seeded into both homozygous ARQ and AHQ substrates.

Interestingly and contrary to classical scrapie and CH1641, the three homozygous ARQ Ovine BSE samples were successfully amplified and detected in all amplification experiments independent of the *Prnp* substrate genotype or the volume of spike seeded into the PMCA reaction or the number of PMCA rounds. It seems that ovine BSE conformers are compatible with multiple conformations assumed by the different substrate genotypes, and are readily able to amplify and produce similar stable conformers that support further substrate conversion. In contrast, classical scrapie and CH1641 strains are not capable of forming some of these stable conformers and their amplification is much more dependent on other variables

affecting amplification *in vitro*. *In vivo* studies showed that BSE and CH1641 can infect homozygous VRQ, ARQ and AHQ sheep (Hunter, 2003, Goldmann *et al*., 1994). Furthermore, since the first isolation of CH1641 isolate in 1970, it is maintained *in vivo* through sub-passage in sheep of different *Prnp* genotypes. Therefore, why ovine BSE and not CH1641 prions would be efficiently amplified in this study is not clear. These phenomena of prion strain propagation in sheep indicate that the *in vitro* replication for BSE and CH1641 strains may be distinct from *in vivo* propagation. The presented data would suggest that BSE is much more likely to efficiently replicate in sheep with a range of genotypes compared to classical scrapie or CH1641. Yet to date, no BSE infections have been detected in the European sheep population despite extensive surveillance (Stack *et al*., 2006, Lezmi *et al*., 2004). This indicates that there are other factors controlling the spread of infection of these distinct strains in the field amongst sheep populations one or more of which has prevented efficient transmission of the BSE agent.

Despite the similar structure between ovine BSE and CH1641 PrP^{Sc}, CH1641 had very distinct amplification properties. CH1641 was refractory to amplification in VRQ substrate whatever the volume of PrP^{Sc} spike or PMCA rounds. This distinct pattern of amplification compared to ovine BSE is not due to the level of protease resistant PrP^{Sc} within the seed. For example, ovine BSE (1693/03) and CH1641 (J2916) contained low levels of PrP^{Sc}, whereas CH1641 (J2935, J3011 and VLA-1) samples, together with ovine BSE (0654/04 and 0392/04) contained relatively high levels of PrP^{Sc} (Chapter 4, Figure 4.1)

When matching template-substrate interaction for *Prnp* genotype, neither of the two homozygous AHQ CH1641 samples nor the two ARQ/AHQ CH1641 samples amplified in AHQ substrate after 5 rounds of PMCA reaction. However ovine BSE (ARQ/ARQ isolates) were readily amplified in the AHQ substrate even after 1 round of sPMCA. These data strongly indicate that the success of amplification seen for ovine BSE and CH1641 are due to prion strain and not to *Prnp* genotype effects.

The distinct amplification patterns for BSE and CH1641 isolates in VRQ and AHQ substrates over 5 rounds of PMCA reactions have now been validated in independent experiments and could form the basis for a rapid strain typing assay.

My results demonstrate that PrP^{res} can be formed following spiking ARQ substrate and performing PMCA reaction. However, the pattern of PrP^{res} production was variable in the amount of PrP^{res} produced from some seeded spikes at different rounds. PrP^{res} production peaked at 3 - 5 rounds of amplification and subsequent rounds contained less PrP^{res} and PrP^{res} was not detectable at later rounds for some experiments. Previous studies of prion propagation in a cell culture model showed fluctuation in PrP^{res} level that was dependent on composition of the medium and the differentiation state of cells, cells density and direct cell to cell contact (Nakamitsu *et al*., 2010, Ghaemmaghami *et al*., 2007, Bate *et al*., 2004, Rubenstein *et al*., 1990). Furthermore, the acute formation and persistence of PrP^{res} in cell lines might depend upon the scrapie strains ability to persistently infect the cellular compartment and in turn the sustainable formation of PrP^{res} (Vorberg *et al*., 2004). Therefore, using PMCA as a cell free conversion assay with ARQ substrate, whilst 10 out of 16 scrapie isolates were amplified, the products were often only seen after high numbers of rounds or were produced transiently with low levels or absence of PrP^{res} at later rounds. I speculate that for the latter isolates the PrP^{res} formation and propagation was driven mainly by the PrP^{Sc} present in the spike and that whilst PrP^{Sc} conformers within each spike were readily able to amplify rapidly, the PrP^{res} products of amplification could not support further efficient recruitment of PrP^{C} substrate and formation of PrP^{res}. Similar results were found using cell culture, where acute formation of PrP^{res} was not indicative of a persistent scrapie infection (Vorberg *et al*., 2004). In addition, if replication of prions was relatively inefficient, it might be that the methodology of diluting amplified products 1: 3 into fresh PrP^{C} substrate between each round reduced the quantity of *in vitro* produced PrP^{Sc} available to act as template for further conversion events. Alternatively, there is potential that amplification with ARQ substrate produced PrP^{Sc} conformers that were more sensitive to protease digestion which therefore could not be detected after PK digestion. These scenarios may well result in apparently transient or low efficiency production of PrP^{Sc} during sPMCA. This distinct pattern of scrapie amplification within ARQ substrate appears to be very much dependent on the individual spike and not the scrapie strain.

Collectively, although not all strains have been amplified in this study due to the complex nature of ovine prion diseases, PMCA is still a promising diagnostic

methodology of high sensitivity. It is also a useful technique for studying prion propagation. The data obtained in these preliminary *in vitro* amplification experiments might correlate with my knowledge and understanding to genotype/species barriers phenomena in classical scrapie transmission *in vivo,* where VRQ sheep are the most susceptible genotype to infection in comparison to ARQ and AHQ sheep. Based on various animal experimental studies, the species barrier or genotype barrier is thought to be a combination of different factors that include differences in primary amino acid sequence between the donor and host, in the glycoform patterns, and in PrP three dimensional structures which can result in inefficient interaction between donor PrP^{Sc} and host PrP^{C} (Moore *et al*., 2005, Hill *et al*., 2000). Moreover, the effect of a variety of different prion strains has been found (Jeffrey *et al*., 2006a, Telling, 2004, Bruce, 2003, Houston *et al*., 2002).

A major new finding in my study is the potential of using sPMCA as a strain typing method for differentiating between two prion strains with similar molecular profiles, such as CH1641 and ovine BSE. Further investigation looking at repeat analysis of CH1641 and BSE samples as well as using panels of CH1641-like field isolates will be of high importance in validating this strain typing methodology.

Chapter 6. *In Vitro* Amplification of Different PrP^{Sc} Strains in rPrP Substrates.

6.1. Introduction:

The initial success of sPMCA to detect extremely low levels of PrP^{Sc} in brain homogenates, secreta and excreta of scrapie infected animals indicates the potential for this method in prion disease diagnosis and research. However, a major limitation is imposed by the use of crude brain homogenate as a substrate. This method requires a consistent supply of brain tissue from scrapie free sheep that imposes both a financial burden and technical difficulties. In addition, variations within brain homogenate substrate of the same *Prnp* genotypes have been demonstrated to affect amplification efficiency (B. Maddison, personal communication). These disadvantages have created the need to circumvent the use of brain homogenates as substrate. In addition, as demonstrated in the previous chapter the PrP^{Sc} genotype and prion strain can affect amplification efficiencies. Therefore, a single substrate that would support PMCA amplification with little or no influence of prion strain or genotype is still a requirement if PMCA is to be applied for diagnosis of prion diseases in small ruminant. One possibility to overcome the limitation of PMCA is by using rPrP expressed in bacteria as a substrate, known as rPrP- PMCA.

rPrP expressed in *Escherichia coli* has been used as a substrate for conversion into distinct conformers with some properties of PrP^{Sc}. This model has been used to understand different aspects of prion conversion including the self-replicating properties and species-specificity of *in vitro* fibrillization reactions; particularly using human, hamster and mouse models (Baskakov, 2009, Makarava *et al*., 2009, Moore *et al*., 2009, Lee *et al*., 2007, Surewicz *et al*., 2006, Bocharova *et al*., 2005, Baskakov, 2004, Baskakov *et al*., 2002). Furthermore, *in vitro* studies have looked at the physico-chemical properties of rPrP allelic variants of sheep (Rezaei *et al*., 2000), particularly with respect to prion strain specific effects on PrP conversion (Kupfer *et al*., 2007, Eiden *et al*., 2006), to gain further insight into the sequence linked basis of scrapie susceptibility and resistance (Morel *et al*., 2007). Moreover, the conversion mechanism of recombinant bovine PrP to amyloid fibrils using BSE-prion as seeds and producing PrP^{Sc}-seeded fibrils has been shown to be a model of *in vivo* BSE infection, adding to the understanding of a significant veterinary disease (Panza *et al*., 2008). Additionally, recombinant ovine and murine PrP were used to simulate intra- and interspecies transmission *in vitro* (Panza *et al*., 2010).

Recent studies have illustrated the use of bacterially expressed rPrP as a substrate in cell-free conversion assays (Peden *et al*., 2012, Orrú *et al*., 2011, Wang *et al*., 2011, Kim *et al*., 2010, Wang *et al*., 2010, Wilham *et al*., 2010, Orru *et al*., 2009, Atarashi *et al*., 2008, Atarashi *et al*., 2007). These studies have shown that the sPMCA procedure can be adapted to convert bacterially expressed recombinant full length Syrian hamster prion protein (rSha PrP) to PK rPrP^{res}, with a 16-17 kDa PK PrP^{res} fragment being generated. Overall, these studies have been limited to a few isolates of rodents, ovine and human TSEs, but the results of these studies have shown promising diagnostic and screening methodologies. They have demonstrated a greater sensitivity, relative simplicity and more rapid detection of minute amounts of PrP^{Sc} in biological samples, including from pre-clinical cases, compared to the use of brain homogenate substrate. However, one challenge with the use of rPrP as the substrate for *in vitro* replication of sheep scrapie is the existence of multiple strains within different genotypes.

Therefore, the aim in this chapter was to assess the use of rSha PrP and ovine VRQ rPrP as substrates in PrP^{Sc} seeded sPMCA reactions with a range of sheep prion seeds to generate PK rPrP^{res} fragments. The future aim being that this *in vitro* replication format will further be validated for the potential application of this system for the development of a pre-clinical prion diagnostic test.

6.2. Results:

6.2.1. Expression and purification of full length rPrP:

Ovine PrP (PrP23-231), containing VRQ polymorphism, was expressed within *E. coli* resulting in the production of rPrP within insoluble cytoplasmic inclusion bodies. These inclusion bodies containing highly purified rPrP had shown approximately equal quantities of both full length and truncated PrP fragments (Figure 6.1). In order to separate full length rPrP from other truncated PrP fragments, a purification strategy was employed to take advantage of the metal chelating capacity of the octapeptide repeat region (Viles *et al*., 1999) through direct binding to Cu ions. FPLC affinity purification included denaturation of the protein, binding to Cu-containing resin, refolding of the full length rPrP through a wash step using a decreasing urea gradient, and elution with imidazole. The pooled protein fractions

corresponding to a peak elution on the A280 chart recorder showed a band of single species with an estimated molecular mass of approximately 24 kDa when analysed by Coomassie staining of SDS-PAGE gels of the eluted fraction (Figure 6.1). This represents full length PrP with no PrP fragment contamination. Quantification of rPrP using a Bradford assay against a BSA standard curve indicated that the concentration of rPrP was 0.5 mg/ml in a total volume of 13 ml (data not shown). The total quantity of rPrP recovered was therefore 6.5 mg. This represented the purification from 500 ml of the original expression culture; therefore, the yield of pure, soluble rPrP obtainable per litre of bacterial culture would be approximately 13 mg. The same procedures were applied to expression and purification of full length rSha PrP.

6.2.2. Optimization of rPrP substrate concentration and number of sPMCA rounds:

rPrP substrate concentration was optimised through sPMCA reactions seeded with 50µg of caudal medulla brain homogenate from VRQ/VRQ classical scrapie affected sheep (1563/02) using two concentrations of monomeric rSha PrP and VRQ rPrP substrates; 0.2 mg/ml and 0.1 mg/ml of each. Upon completion of the reaction (mainly 3 – 6 rounds), the sonicated products were digested with PK and analysed by gel electrophoresis and Western blotting. The concentration of PK used was 10 µg/ml based on the results of optimising PK concentration for digestion previously described (Chapter 2: Materials and methods and Figure 6.2).

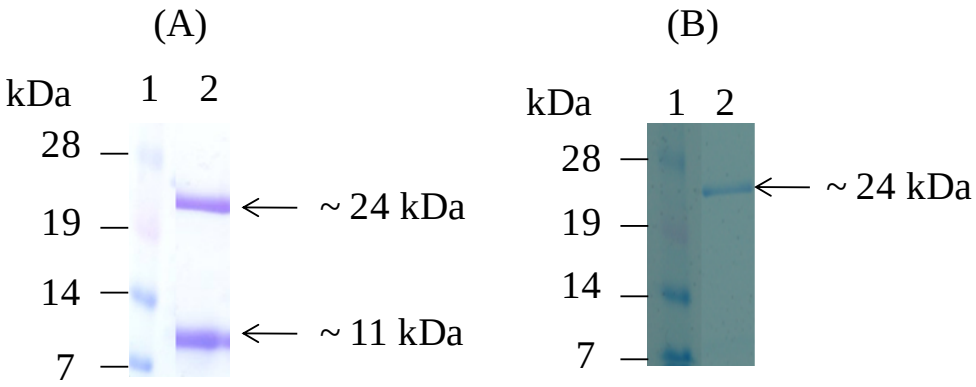


Figure 6.1: Production of pure rPrP: PrP was expressed in *E. coli* and washed inclusion bodies resolubilised in urea were analysed. The SDS-PAGE and Coomassie Brilliant Blue staining of the solubilised inclusion bodies containing rPrP showed both full length (approximately 24 kDa) and truncated PrP fragments (approximately 11 kDa) (A: lane 2). After FPLC purification that involved rPrP being purified on an immobilised metal affinity column, full length rPrP (approximately 24 kDa) was obtained with no PrP fragment contamination (B: lane 2).

Primarily, when samples were probed with SAF84, no signals of PK $rPrP^{res}$ fragments were produced from spiked samples in 0.1 mg/ml substrate (both rSha PrP and VRQ rPrP) following 2 rounds of amplification. After 3 rounds, signals were produced with a band of approximately 7 - 8 kDa. After 6 rounds of amplification, the signal produced had increased in intensity and further band of approximately 16 kDa (together with bands of ~ 7 - 10 kDa) were detected (Figure 6.3).

The analysis of sPMCA products from spiked samples in 0.2 mg/ml substrate showed no detectable PK $rPrP^{res}$ fragments after the first round of PMCA reaction using both rSha PrP and VRQ rPrP substrates (data not shown). After the second round, there were amplified products from scrapie spiked samples in VRQ rPrP substrate with clear PK $rPrP^{res}$ fragments of 7-8 kDa and 16 kDa, while no fragments were detected from samples spiked in rSha PrP (data not shown). Analysing samples from the 3^{rd} round showed clear bands of PK $rPrP^{res}$ fragments produced from spiked samples in both substrates. Further rPrP-PMCA reaction using both rSha PrP and VRQ rPrP substrate spiked with multiple classical scrapie affected caudal medulla samples (VRQ/VRQ 1563/02, VRQ/VRQ 1275/02, VRQ/VRQ 1276/02) and VRQ/VRQ (1699/05) healthy cerebellum as a control, showed efficient amplification of all scrapie spikes (Figure 6.4). Given the apparent increase in sPMCA efficiency when using 0.2 mg/ml VRQ PrP substrate, a concentration of 0.2 mg/ml substrate was selected for further analysis for the generation of PK $rPrP^{res}$ fragments using rSha PrP and VRQ rPrP substrates.

6.2.3. Optimization of anti-PrP antibodies for the discrimination of PrP^{Sc} and PrP^{C} spiked samples following PMCA amplification:

To detect PK-resistant fragments $rPrP^{res\ (Sc)}$ (resulting from seeding reactions with either 50 µg brain homogenate scrapie affected medulla (1563/02), or 50 µg healthy cerebellum homogenate (1699/05), and to specifically differentiate between scrapie and healthy spiked samples, PK resistant rPrP fragments were probed with panels of antibodies.

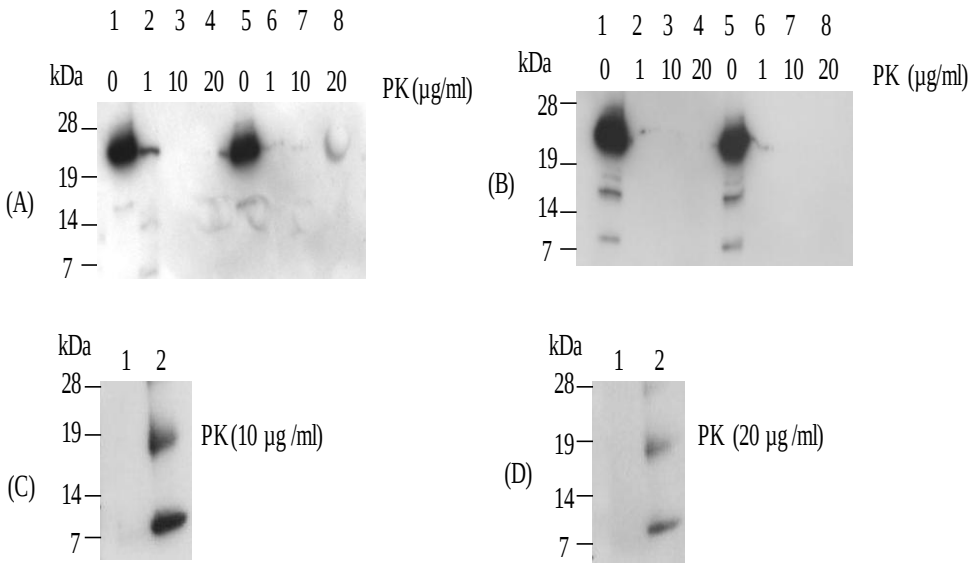


Figure 6.2: Western blot of the optimization of PK concentration for the digestion of rPrP^{sen} and detection rPrP$^{res\text{-}Sc}$: Panels A and B: 10 µl of 0.1 mg/ml rSha PrP^{sen} (two batches on panel A lanes 1- 4 and 5 - 8) and VRQ rPrP^{sen} (two batches on panel B lanes lanes 1- 4 and 5 - 8) were treated with 0, 1, 10, or 20 µg/ml PK as indicated for 1 h at 37°C. 5 µl of each digestion were loaded per lane. Panels C and D: following 3 rounds of sonication, using 0.2 mg/ml rSha PrP^{sen} substrate (one batch) spiked with either healthy (lane 1) or scrapie brain homogenate (lane 2) were digested with 10 µg/ml PK (panel C) or 20 µg/ml PK (panel D). All Digested samples were analysed by gel electrophoresis and immunoblotting with SAF84 antibody (1: 40,000).

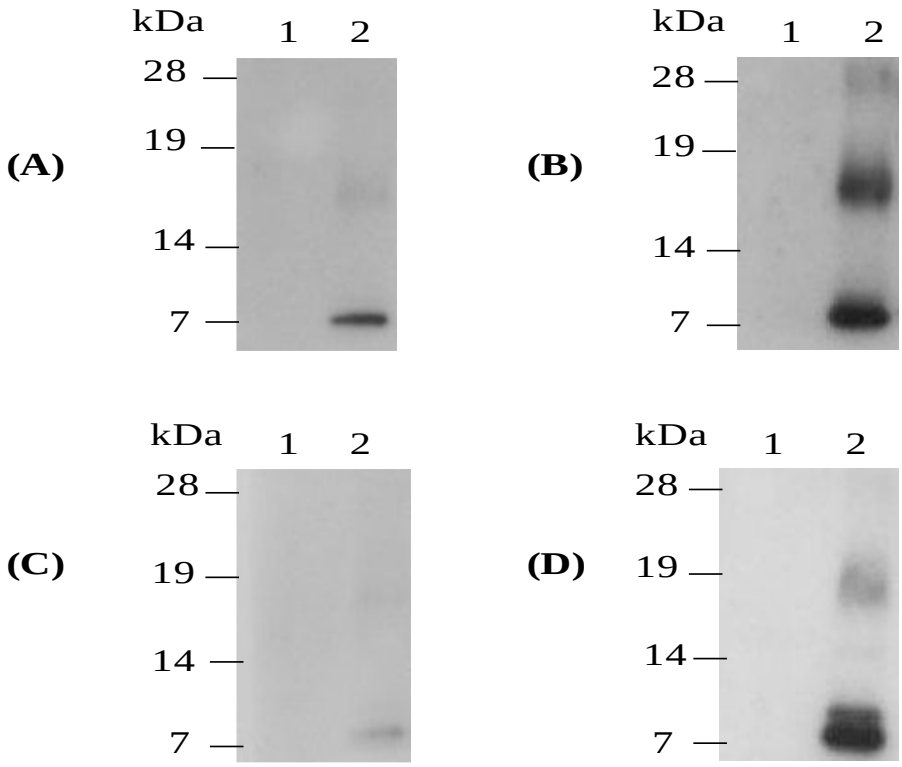


Figure 6.3: Western blot of the optimization of rPrP substrate concentration and number of sPMCA rounds: 50 µg spike of classical scrapie affected brain homogenate (VRQ/VRQ, 1563/02) was seeded into 85 µl of 0.1 mg/ml rPrP substrates; rSha PrP (A & B) and VRQ rPrP (C & D). Both seeded substrates were subjected to 3 rounds (A & C) and 6 rounds (B & D) of sPMCA reaction. Sonicated samples were digested for 1 hour at 37ºC with 10 µg/ml PK and 5 µl of each digested sample was analysed by Western blot. PK $rPrP^{res}$ fragments were detected with mAb SAF84 (1: 40,000). Lane 1: ovine classical scrapie (VRQ/VRQ, 1563/02) spiked sample without sonication, Lane 2: ovine classical scrapie (VRQ/VRQ, 1563/02) spiked sample after sonication. Molecular weight markers are shown (kDa).

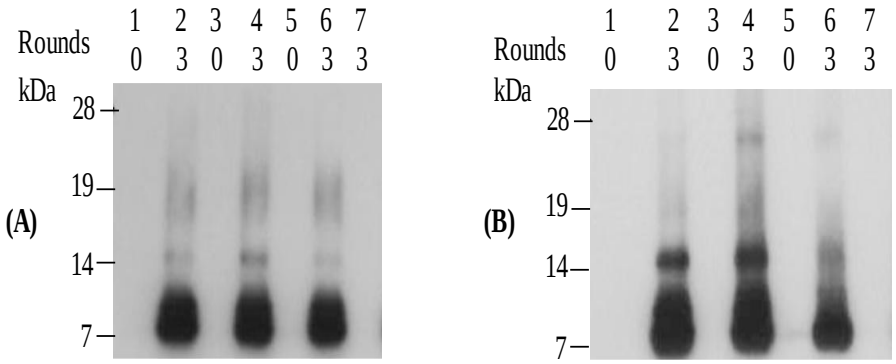


Figure 6.4: Western blot of the optimization of rPrP substrate concentration (0.2 mg/ml) and number of sPMCA rounds: 50 µg spike of VRQ/VRQ classical scrapie affected caudal medulla homogenates from 3 different animals (Lane 1 & 2: 1563/02, lane 3&4: 1275/02, lane 5 & 6: 1276/02), together with 50 µg spike of healthy caudal medulla homogenate (lane 7: 1699/05) were seeded into 85 µl of 0.2 mg/ml rPrP substrates; rSha PrP (A) and VRQ rPrP (B) in duplicate. Both seeded substrates were subjected to two treatments; one set of seeded samples was frozen immediately after spiking and called round 0. The second set of same seeded samples were subjected to 3 rounds of sPMCA reaction and called round 3. Both round 0 and round 3 sonicated samples were digested for 1 hour at 37ºC with 10 µg/ml PK and 10 µl of each digested sample were analysed by Western blot. PK $rPrP^{res}$ fragments were detected with mAb SAF84 (1: 40,000). Molecular weight markers are shown (kDa).

These antibodies recognise different epitopes directed towards either the N- or C-terminal of PrP; AG4, SAF32, P4, 8G8, SAF70, SHA31, SAF84 and 8H4. These monoclonal antibodies were diluted and used to probe Western blots (Chapter 2, Materials and methods, Table 2.1).

No PK rPrP^{res} products were detected when SAF32, P4, 8G8 and 8H4 antibodies were used. When samples were probed with AG4 (diluted 1: 2,000), a single or double band with a molecular mass of ~ 7 - 9 kDa was the marker for the presence of PK-resistant fragments produced from PrP^{Sc} spiked rSha PrP substrate (Figure 6.5: A). Using either SAF70 or SAF84 (both were diluted 1: 40,000), a PK-resistant fragment with a molecular mass of approximately 7 kDa was detected from PrP^{Sc} seeded reactions. However, a relatively weak band of ~ 7 kDa was also seen in some PrP^{C} seeded reactions (Figure 6.5: B). This weak band also occurred with non-seeded sonicated control samples (data not shown). In addition, a further band of PK-resistant fragments of approximately 17-19 kDa was produced specifically from PrP^{Sc} seeded reactions (Figure 6.5: B and C). However, when the same PK-resistant fragments were subjected to immunoblotting with anti-PrP antibody SHA31 (diluted 1: 80,000) a set of PK-resistant fragments with molecular mass of ~10, 12, and ~15 kDa were detected in PrP^{Sc} seeded reaction. No samples seeded with healthy brain homogenates or non-spiked control substrate samples subjected to sPMCA generated any PK-resistant, SHA31 reactive fragments (Figure 6.5: D). Therefore, to further examine the specificity of mAbs to differentiate between scrapie and healthy spiked samples, further PMCA reactions seeded with range of classical scrapie, CH1641 scrapie and ovine BSE samples were carried out, and PK-resistant fragments were detected using SAF70 or SHA31.

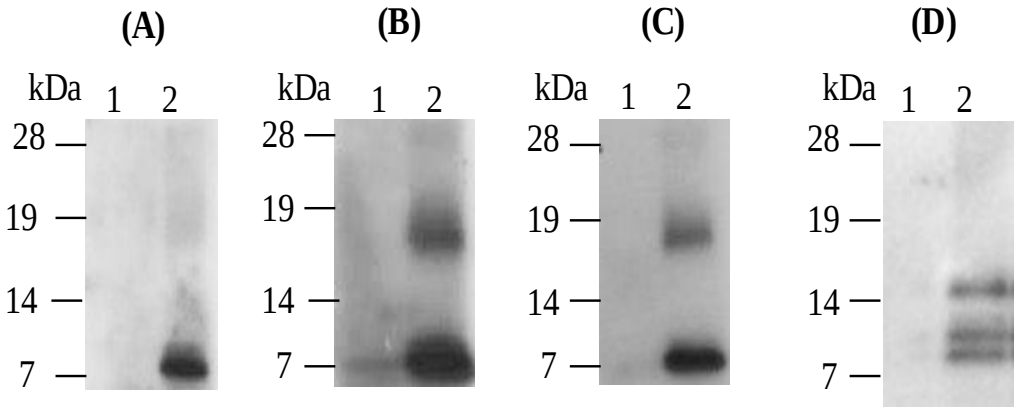


Figure 6.5: Western blot of the comparison of antibodies to detect PMCA products following amplification of PrP^{Sc} and PrP^{C} spiked samples: 50 µg spike of healthy brain homogenate (Lane 1: VRQ/VRQ, 1699/05) or classical scrapie affected brain homogenate (Lane 2: VRQ/VRQ, 1563/02) were seeded into 85 µl rSha PrP and subjected to 3 rounds of sPMCA. Sonicated samples were digested for 1 hour at 37ºC with 10 µg/ml PK and 10 µl of each digested sample was analysed by Western blot. PK rPrP fragments were detected with monoclonal antibodies: (A) AG4 (1: 2,000), (B) SAF70 (1: 40,000), (C) SAF84 (1: 40,000) and (D) SHA31 (1: 80,000). Molecular weight markers are shown (kDa).

6.2.4. The application of rPrP-PMCA to detect different strains of prion diseases in sheep:

Reactions were seeded with 50 µg of infected cerebellum homogenate for classical scrapie, CH1641 scrapie and ovine BSE, or with 50 µg of healthy ovine cerebellum homogenate. Isolates analysed were from sheep with a range of homozygous and heterozygous *Prnp* genotypes (Table 6.1). Amplifications were carried out in both rSha PrP and VRQ rPrP [0.2 mg/ml rPrP in conversion buffer] substrates. The reactions were carried out according to the sPMCA protocol described previously (Chapter 2: Materials and Methods). Three to four sPMCA rounds were performed for each sample. Further samples were prepared and analysed in exactly the same way as for the sPMCA samples, but were to act as controls without being subjected to amplification.

The initial analysis of the products of amplifications of a range of classical scrapie, ovine BSE and CH1641 isolates (n = 19) along with healthy control (n = 4) and non-seeded sonicated control sample (1) with mAb SAF70, showed a PK-resistant fragment with a molecular mass of ~ 7 kDa that was detected from PrP^{Sc} seeded reactions, as well as healthy seeded and non-seeded control samples. Moreover, a further band of PK-resistant fragments of approximately 17-19 kDa was produced from some but not all PrP^{Sc} seeded reactions (10 out of 19 reactions in duplicates) (data not shown). Further analysis of the same products of amplification with mAb SHA31 revealed PK-resistant fragments with molecular mass of either ~10, or ~ 12, or ~15 kDa for PrP^{Sc} seeded reactions. Some PrP^{Sc} seeded reactions showed the formation of all 3 of $rPrP^{res(Sc)}$ bands. Neither samples seeded with healthy brain homogenates nor non-spiked sonicated control substrate samples subjected to sPMCA generated any PK-resistant fragments. Based on the results of SAF70 in detecting PK-resistant fragments, it seems that there are spontanous generation of PK- resistant fragments of 7 kDa from all seeded and non-seeded samples as a result of sonication.

Therefore, in all future applications it was decided to detect all rPrP-PMCA products with SHA31and if any single band of one of the 3 $rPrP^{res(Sc)}$ specific bands was detected, the sample would be considered successfully amplified.

Table 6.1: Amplification of ovine cerebellum brain homogenate of different *Prnp* genotypes by sPMCA using recombinant substrate.

Isolate ID	genotype	strain	rSha PrP[a]		VRQ rPrP[a]	
			R3[b]	R4[b]	R3[b]	R4[b]
1563/02	VRQ/VRQ	Classical Scrapie	2	-[c]	2	2
1276/02	VRQ/VRQ	Classical Scrapie	2	-	2	2
1275/02	VRQ/VRQ	Classical Scrapie	2	-	2	2
0923/03	VRQ/VRQ	Classical Scrapie	2	-	2	2
0925/03	VRQ/VRQ	Classical Scrapie	2	-	2	2
0836/03	VRQ/VRQ	Classical Scrapie	2	-	2	2
SSBP1	VRQ/VRQ	Classical Scrapie	2	-	2	2
0226/03	ARQ/VRQ	Classical Scrapie	2	-	2	2
0455/03	ARQ/VRQ	Classical Scrapie	2	-	2	2
0615/03	ARQ/VRQ	Classical Scrapie	2	-	2	2
0678/03	ARQ/ARQ	Classical Scrapie	2	-	2	2
0635/03	ARQ/ARQ	Classical Scrapie	2	-	2	2
J2916	ARQ/AHQ	CH1641	2	-	2	2
J2935	ARQ/AHQ	CH1641	2	-	2	2
J3011	AHQ/AHQ	CH1641	2	-	2	2
VLA1	AHQ/AHQ	CH1641	0	2	2	2
1693/03	ARQ/ARQ	Ovine BSE	0	0	1	2
0654/04	ARQ/ARQ	Ovine BSE	2	-	2	2
0392/04	ARQ/ARQ	Ovine BSE	0	1	0	2
0877/03	ARQ/ARQ	Healthy ovine	0	0	0	2
1035/03	ARQ/ARQ	Healthy ovine	0	1	0	0
1037/03	ARQ/ARQ	Healthy ovine	0	0	0	0
1699/05	VRQ/VRQ	Healthy ovine	0	0	0	0
C	rPrP substrate	substrate only (no spike)	0	0	0	0

[a] PMCA substrate, both at 0.2 mg/ml.
[b] round of amplification, each sample was amplified in duplicate and the numbers of positive reaction are indicated (as detected with SHA31).
[C] -; not done.

6.2.4.1. Amplification of ovine prions seeded into VRQ rPrP-PMCA reactions:

After 3 rounds of amplification, VRQ rPrP (0.2 mg/ml) reactions seeded with PrP^{Sc} showed successful amplification, with the detection of PK $rPrP^{res(Sc)}$ bands of approximately 10 and 12 kDa, and on occasion, further bands of 15 kDa when immunoblotted with the SHA31 antibody. PK $rPrP^{res(Sc)}$ were detected in 35 out of 38 reaction. The samples that were not successfully amplified were one of the duplicate samples of ovine BSE (ARQ/ARQ, 1693/03) and both duplicate samples of ovine BSE (ARQ/ARQ, 0392/04). There was no PK-resistant product generated from the reactions seeded with brain homogenate spikes from 4 healthy sheep, or non-seeded rPrP substrate (10 samples) (Table 6.1 and Figure 6.6).

To enhance the detection and production of $rPrP^{res(Sc)}$, samples which gave no PK-resistant product after 3 rounds of amplification were subjected to a further round of sPMCA sonication. This resulted in all TSE-spiked samples generating PK-resistant fragments. However, this also resulted in the generation of PK-resistant bands from one healthy brain homogenate spike (ARQ/ARQ, 0877/03). These PK-resistant fragments produced from reactions seeded with brain homogenates from healthy sheep, or in non-seeded rPrP substrate were considered to be spontaneous PK-resistant fragments, called $rPrP^{res(spon)}$.

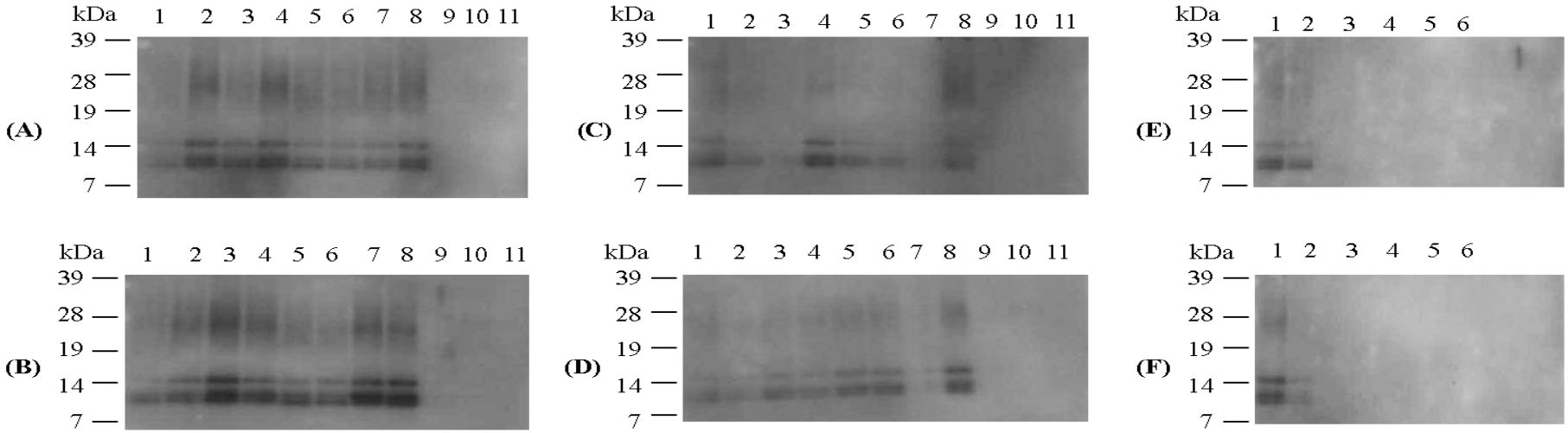


Figure 6.6: Western blot molecular characterization of VRQ rPrP PMCA seeded reactions: 50 µg of each classical scrapie, CH1641 and ovine BSE (19 spikes in duplicates) together with 8 samples each seeded with 50 µg healthy brain homogenates from 4 sheep were subjected to sPMCA reaction (Table 6.1). Each seeded reaction was within 85 µl VRQ rPrP (at 0.2 mg/ml) and was for 3 rounds. Sonicated samples were digested with PK (10 µg/ml) and analysed by Western blotting. Bound PK $rPrP^{res}$ were probed with SHA31 anti-PrP antibody (1: 80,000) and goat anti-mouse HRP conjugate diluted (1: 40,000) and visualised after exposure of the membranes on Fuji X- ray film. A and B: Lane 1: ARQ/VRQ (0226/03), Lane 2: VRQ/VRQ (1563/02), Lane 3: VRQ/VRQ (1276/02), Lane 4: VRQ/VRQ (1275/02), Lane 5: VRQ/VRQ (0923/03), Lane 6: VRQ/VRQ (0925/03), Lane 7: ARQ/VRQ (0455/03), Lane 8: VRQ/VRQ (0836/03), Lane 9: Healthy BH (0877/03), Lane 10: (A): unamplified VRQ/VRQ (SSBP1) (round 0), (B): unamplified ARQ/ARQ (0678/03) (round 0), Lane 11: digested VRQ rPrP substrate. C and D: Lane 1: ARQ/VRQ (0615/03), Lane 2: VRQ/VRQ (SSBP1), Lane 3: ARQ/AHQ (CH1641, J2916), Lane 4: ARQ/AHQ (CH1641, J2935), Lane 5: AHQ/AHQ (CH1641, J3011), Lane 6: AHQ/AHQ (CH1641), Lane 7: ovine BSE, ARQ/ARQ (1693/03), Lane 8: ovine BSE, ARQ/ARQ (0654/04), Lane 9: Healthy BH (1035/03), Lane 10: (A): unamplified ARQ/AHQ (CH1641, J2935), (round 0), (B): unamplified ARQ/ARQ (0392/04), (round 0), Lane 11: VRQ rPrP substrate. E and F: Lane 1: ARQ/ARQ (0678/03), Lane 2: ARQ/ARQ (0635/03), Lane 3: ovine BSE ARQ/ARQ (0392/04), Lane 4: Healthy BH (1037/03), Lane 5: Healthy BH (1699/05), Lane 6: control sample (non-seeded sonicated rPrP substrate only).

6.2.4.2. Determining the sensitivity and specificity of sPMCA for the detection of PrP^{Sc} using VRQ rPrP substrate with two different concentrations:

The level of sensitivity of VRQ rPrP PMCA reaction for the detection of PrP^{Sc} was carried out through eight 10x times dilution series of VRQ/VRQ classical scrapie affected brain homogenate (0836/03) starting with 50 µg of brain in duplicate. In addition, to test specificity, 20 negative control reactions, each seeded with 0.5 µg brain homogenate from 8 healthy sheep with different *Prnp* genotypes were also analysed. The level of sensitivity of VRQ rPrP PMCA reaction was carried out using different VRQ rPrP substrate preparations in two different independent experiments.

When different batches of VRQ rPrP substrate were used (final concentration 0.2 mg/ml), $rPrP^{res(Sc)}$ was detected in all duplicate samples seeded with PrP^{Sc} from as little as 0.5 ng of brain. Whilst no PK $rPrP^{res}$ was observed in 7 negative control samples seeded with healthy brain homogenate representing 3 healthy animals (ARQ/ARQ, 1037/03, VRQ/VRQ, 1699/05, AHQ/AHQ, 1102/05), $rPrP^{res(spon)}$ was detected from other negative spiked samples from healthy animals either in both duplicate samples (ARQ/ARQ, 0877/03) or one of two samples (ARQ/ARQ, 1035/03, VRQ/VRQ, 1100/05 and AHQ/AHQ, 1101/05) or two of the triplicate samples (VRQ/VRQ, 1111/05). Furthermore, PK $rPrP^{res(spon)}$ was also detected in one of the duplicate non-seeded rPrP substrate samples (Figure 6.7).

Using the same VRQ rPrP batch, but at a decreased concentration of 0.1 mg/ml, PK $rPrP^{res(Sc)}$ was detected in all duplicate samples seeded with PrP^{Sc} from as little as 50 ng of brain and no PK $rPrP^{res(spon)}$ from the reaction seeded with healthy brain homogenates or non-seeded rPrP substrate were observed by Western blot (22 amplifications, Figure 6.8, Table 6.2).

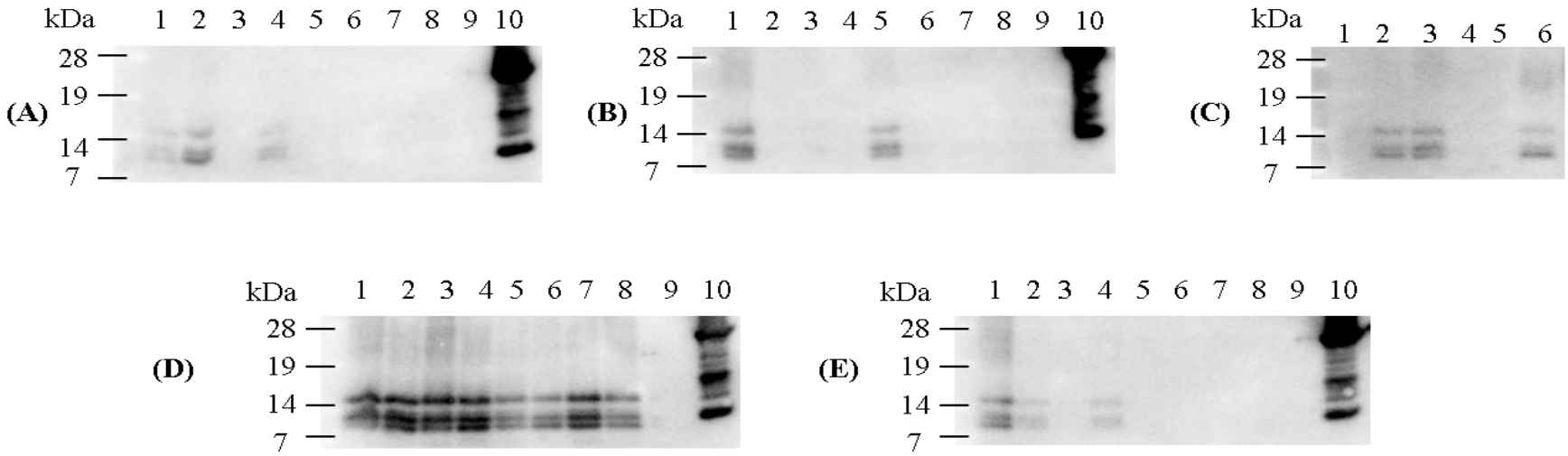


Figure 6.7: Sensitivity and specificity of sPMCA for the detection of PrP^{Sc} using VRQ rPrP substrate (batch 2 at concentration of 0.2 mg/ml): 50 µg of each eight 10x dilution series of VRQ classical scrapie (0836/03), together with 20 samples each seeded with 50 µg of healthy brain homogenates from 8 sheep with different *Prnp* genotypes were spiked into 85 µl VRQ rPrP (0.2 mg/ml) and subjected to 3 rounds of sPMCA reaction. Sonicated samples were digested with PK (10 µg/ml) and analysed by Western blotting. PK $rPrP^{res}$ were probed with SHA31 anti-PrP antibody (1: 80,000) and goat anti-mouse HRP conjugate diluted (1: 40,000) and visualised by using an ICCD225 camera . A, B and C: Lanes 1 - 9 show amplifications containing no PrP^{Sc}. A: Lane 1 & 2: ARQ/ARQ (0877/03), Lane 3 & 4: ARQ/ARQ (1035/03), Lane 5 & 6: ARQ/ARQ (1037/03), Lane 7 & 8: VRQ/VRQ (1699/05). B: Lane 1, 2 & 3: VRQ/VRQ (1100/05), Lane 4, 5 & 6: AHQ/AHQ (1101/05), Lane 7, 8 & 9: AHQ/AHQ (1102/05). C: Lane 1, 2 & 3: VRQ/VRQ (1111/05), Lane 5 & 6: VRQ rPrP substrate only. D and E show amplifications of the dilution series of a scrapie affected ovine brain in duplicates. D: Amplifications containing 10x dilution series of 50,000 to 50 ng of brain is shown in lanes 1 & 2, 3 & 4, 5 & 6, 7 & 8 respectively. E: Amplifications containing 10x dilution series of 5 to 0.005 ng of brain is shown in lanes 1 & 2, 3 & 4, 5 & 6, 7 & 8 respectively. For panels A, B, D and E, lane 10 contains 3 µl undigested VRQ rPrP substrate. Molecular weight markers are shown (kDa).

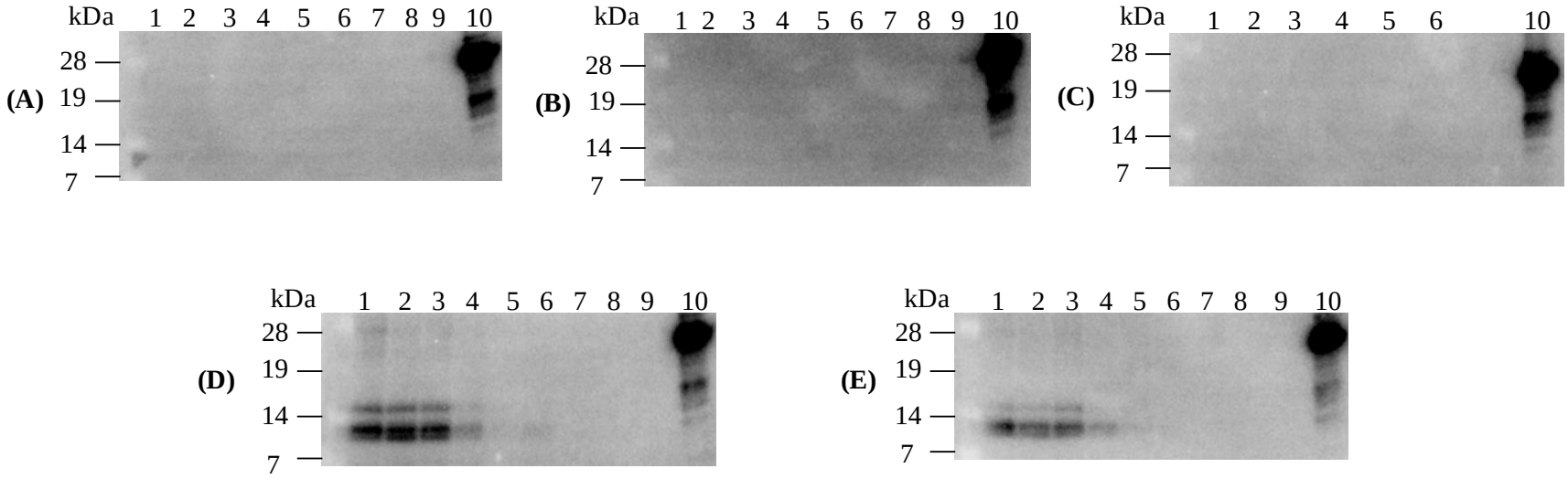


Figure 6.8: Sensitivity and specificity of sPMCA for the detection of PrP^{Sc} using VRQ rPrP substrate (batch 2 at concentration of 0.1 mg/ml): 50 µg of each eight 10x dilution series of VRQ classical scrapie (0836/03), together with 20 samples each seeded with 50 µg of healthy brain homogenates from 8 sheep with different *Prnp* genotypes were spiked into 85 µl VRQ rPrP (0.1 mg/ml) and subjected to 3 rounds of sPMCA reaction. Sonicated samples were digested with PK (10 µg/ml) and analysed by Western blotting. PK $rPrP^{res}$ were probed with SHA31 anti-PrP antibody (1: 80,000) and goat anti-mouse HRP conjugate diluted (1: 40,000) and visualised by using an ICCD225 camera (Photek Ltd., East Sussex, United Kingdom). A, B and C: lanes 1 - 9 show amplifications containing no PrP^{Sc}. A: Lane 1 & 2: ARQ/ARQ (0877/03), Lane 3 & 4: ARQ/ARQ (1035/03), Lane 5 & 6: ARQ/ARQ (1037/03), Lane 7 & 8: VRQ/VRQ (1699/05). B: Lane 1, 2 & 3: VRQ/VRQ (1100/05), Lane 4, 5 & 6: AHQ/AHQ (1101/05), Lane 7, 8 & 9: AHQ/AHQ (1102/05). C: Lane 1, 2 & 3: VRQ/VRQ (1111/05), Lane 5 & 6: VRQ rPrP substrate only. D and E are replicate analysis of amplifications of the dilution series of a scrapie affected ovine brain. Amplifications containing 50,000 to 0.005 ng of brain are shown in lanes 1 - 8 respectively. For panels A, B, C, D and E, lane 10 contains a 3 µl undigested VRQ rPrP substrate. Molecular weight markers are shown (kDa).

Table 6.2: Variations of specificity and limit of detection of different rSha PrP and VRQ rPrP substrate batches.

Substrate	Batch No.	Substrate concentration	$rPrP^{res(spon)}$ /total, after three rounds of sonication		Limit of Detection (ng) brain
			1st experiment [a]	2nd experiment [b]	
rSha PrP	one	0.2 mg/ml	0/10	0/22	5 ng
rSha PrP	two	0.2 mg/ml	-	0/22	5 ng
VRQ rPrP	one	0.2 mg/ml	0/10	-	-
VRQ rPrP	two	0.2 mg/ml	-	8/22	0.5 ng
VRQ rPrP	two	0.1 mg/ml	-	0/22	50 ng

a 1st experiment: 19 PrP^{Sc} and 4 PrP^{C} spiked isolates (as detailed in table 6.1) together with non-seeded substrate sample (all in duplicates) were subjected to sPMCA reaction.
b 2nd experiment: 8 times dilution series of PrP^{Sc} (in duplicate) together with 20 PrP^{C} spiked samples (representing 8 healthy animals with different genotype) together with two non-seeded substrate samples were analysed.

6.4.2.3. Amplification of ovine prions seeded into rSha PrP-PMCA reactions:

After 3 rounds of amplification, rSha PrP (0.2 mg/ml) seeded reactions supported amplification of 50 µg TSE affected brain homogenate seed in 32 out of a total of 38 PrP^{Sc} spiked reactions (Table 6.1). The samples that were not successfully amplified were CH1641 (AHQ/AHQ, VLA1) and dublicate samples of both ovine BSE (ARQ/ARQ, 1693/03) and (ARQ/ARQ, 0392/04).

Detection of PK $rPrP^{res(Sc)}$ when immunoblotted with SHA31 antibody showed analogous banding patterns similar to VRQ PK $rPrP^{res(Sc)}$ bands. There was no $rPrP^{res(spon)}$ fragments from any of the 8 samples spiked with 50 µg brain homogenate from 4 healthy sheep or non-seeded rPrP substrate (two samples).

After 4 rounds of sPMCA reaction, $rPrP^{res(Sc)}$ was observed in one of the duplicate reactions seeded with ovine BSE spike (0392/04, ARQ/ARQ) and both replicate amplification for CH1641 (AHQ/AHQ, VLA1). However, there was also generation of PK $rPrP^{res(spon)}$ bands resulting from one healthy brain homogenate spike (1035/03, ARQ/ARQ).

6.4.2.4. Determining the sensitivity and specificity of sPMCA for the detection of PrP^{Sc} using rSha PrP substrate:

Similarly to VRQ rPrP, the level of sensitivity of rSha PrP-PMCA reactions was confirmed through seeded reaction of eight 10x dilution series of a VRQ classical scrapie affected brain homogenate (1275/02) starting with 50 µg of brain in duplicate. Again, to test specificity, 20 negative control reactions each seeded with 50 µg healthy brain homogenates from 8 sheep with different *Prnp* genotypes were also subjected to the same conditions of amplification. These amplification reactions were done twice using two different rSha PrP substrate batches to test the effects of rPrP production on amplification efficiency. After 3 rounds of amplification, PK $rPrP^{res(Sc)}$ was detected in all duplicate samples seeded with PrP^{Sc} from as little as 5ng of brain. In addition, no PK $rPrP^{res(spon)}$ products were observed from the reactions seeded with healthy brain homogenates or non-seeded rSha PrP substrate (Figure 6.9). These results were the same in two independent experiments with the two batches of rSha PrP (Table 6.2).

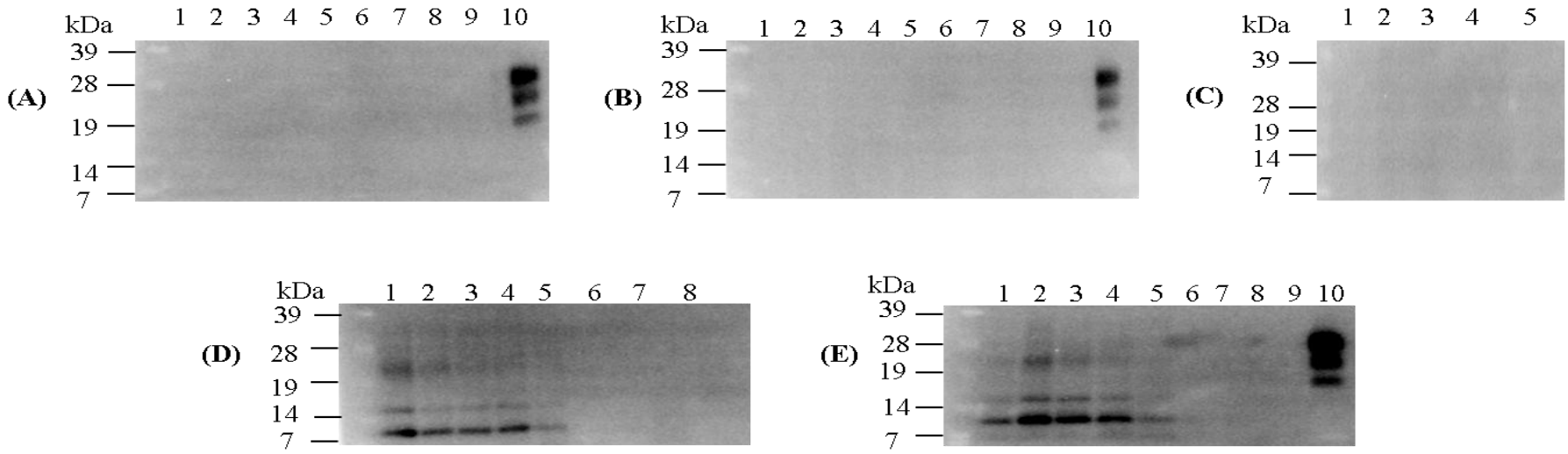


Figure 6.9: Sensitivity and specificity of sPMCA for the detection of PrP^{Sc} using rSha PrP substrate: 50 µg of each eight 10x dilution series of VRQ classical scrapie (1275/03), together with 20 samples each seeded with 50 µg of healthy brain homogenates from 8 sheep with different *Prnp* genotypes were spiked into 85 µl Sha rPrP (0.2 mg/ml) and subjected to sPMCA reaction for 3 rounds. Sonicated samples were digested with PK (10 µg/ml) and analysed by Western blotting. PK $rPrP^{res}$ were probed with SHA31 anti-PrP antibody (1: 80,000) and goat anti-mouse HRP conjugate diluted (1: 40,000) and visualised by using an ICCD225 camera. A, B and C: lanes 1 - 9 show amplifications containing no PrP^{Sc}. A: Lane 1 & 2: ARQ/ARQ (0877/03), Lane 3 & 4: ARQ/ARQ (1035/03), Lane 5 & 6: ARQ/ARQ (1037/03), Lane 7 & 8: VRQ/VRQ (1699/05). B: Lane 1, 2 & 3: VRQ/VRQ (1100/05), Lane 4, 5 & 6: AHQ/AHQ (1101/05), Lane 7, 8 & 9: AHQ/AHQ (1102/05). C: Lane 1, 2 & 3: VRQ/VRQ (1111/05), Lane 4 & 5: rSha PrP substrate only. D and E show replicate amplifications of the dilution series of a scrapie-affected ovine brain. Amplifications containing 50,000 to 0.005 ng of brain are shown in lanes 1 - 8 respectively. For panels A, B and E, lane 10 contains a positive scrapie brain control (digested with 50 µg/ml). Molecular weight markers are shown (kDa).

6.3. Discussion:

One of the major challenges in the TSE field is the development of sensitive, fast and accurate assays for PrP^{res} detection in the pre-clinical diagnosis of TSEs and the identification of potential sources of infection. Therefore, since the advent of the sPMCA method, many reports have shown sensitive detection of PrP^{Sc} at low level in biological and environmental samples. In the present study, to achieve optimal sensitivity and to overcome the limitation of using brain PrP^{C} as an amplification substrate, different trials were conducted using bacterially expressed rPrP protein as a definable and readily available substrate.

The data generated from Western blot analysis of PrP^{Sc} seeded rPrP-PMCA reactions confirm that $rPrP^{res}$ can be generated by sPMCA from highly purified rPrP. Although the rPrP differs from PrP^{C} in the absence of N-linked glycans and GPI moiety, it explains that these cofactors and the post-translational modifications are not essential requirement for prion replication *in vitro*. On the other hand, previous studies also suggested that these cofactors might enhance the production of PrP^{res} *in vitro* as well as *in vivo* (Kim *et al*., 2010 & 2009, Tuzi *et al*., 2008, Chesebro *et al*., 2005). At the same time, rPrP-PMCA generated PK-resistant products are distinct from PrP^{Sc} conformers isolated from scrapie infected brain or PK-resistant PMCA products derived from healthy brain homogenates. This was clearly demonstrated through the lack of glycosylation, differences in molecular weight of $rPrP^{Sc}$ and lower PK resistant PrP^{res} (Smirnovas *et al*., 2009). They are also distinct from amyloid fibres produced during polymerization of rPrP. As visualised by electron microscopy, $rPrP^{Sc}$ are small spherical aggregates rather than elongated fibres (Piro *et al*., 2011).

Orru *et al*., 2009, found that rSha PrP^{sen} was a more effective substrate than ovine rPrP in amplifying ovine classical scrapie using the QuIC reaction conditions. By comparison, I found rSha PrP^{sen} has better specificity than VRQ rPrP at 3 rounds of rPrP-PMCA amplifications, and the specificity of VRQ rPrP varied between batches. Indeed, to achieve high specificity, VRQ needed to be used at a lower concentration than Sha rPrP. The Sha $rPrP^{sen}$ could detect just 5 ng of infected scrapie brain with high specificity. In comparison, VRQ rPrP could detect 50 ng of infected brain with similar specificity. Both substrates demonstrated the same amplification efficiency towards a range of classical scrapie isolates independent of the host *Prnp* genotype.

In contrast, VRQ rPrP (0.2 mg/ml, as demonstrated using batch one) was more efficient preliminary than rSha PrP in amplifying other strains than classical scrapie such as CH1641 and ovine BSE. But, after repeating the sensitivity and specificity test, there was doubt as to the specificity of VRQ rPrP when used at 0.2 mg/ml. These differences in the efficiency of amplification might be related to differences in the amino acid sequence between rSha PrP and VRQ rPrP that might have influence on seed/substrate interaction. It might also be related to other co- factors included in PrP^{Sc} seed contents of these strains.

Under the assay conditions, I could not distinguish between PK $rPrP^{res(Sc)}$ and potentially produced PK $rPrP^{res(spon)}$ from VRQ rPrP (0.2 mg/ml) substrate through the sensitivity towards PK digestion and molecular weight of the resulting PK resistant fragments. They also could not be distinguished by detection with a range of anti-PrP mAbs binding to distinct epitopes (data not shown). This result is in contrast to previous published research work (Smirnovas *et al*., 2009, Atarashi *et al*., 2007). These previous reports demonstrated that a 17 kDa PK-resistant fragment was the most characteristic band for $rPrP^{res(Sc)}$, while $rPrP^{res(spon)}$ had only the smaller 10-11 and 12 kDa when analysed by immunoblotting and probed by R20 hamster PrP reactive anti-sera (directed towards C-terminal residues 219-232), or 12 kDa in the case of polyclonal antibody R 18 (directed towards residue 142-154) (Atarashi *et al*., 2007). Here, using anti-prion rabbit polyclonal antibody with an epitope situated in a very similar region of PrP to that of R20 (raised against a peptide corresponding to residues 217-229 of bovine PrP), I detected a 10 kDa PK-resistant fragment from some but not all healthy-spiked samples as well as from PrP^{Sc} spiked rPrP samples. With monoclonal antibodies SHA31 (directed towards the C-terminal ovine PrP residues 145–152) which binds to a similar region to R18, the molecular weight of PK $rPrP^{res(spon)}$ was indistinguishable to those of PrP^{Sc} seeded sPMCA reactions.

The differences in banding pattern between SHA31 and SAF70 were also difficult to explain. The epitope of SAF70 (directed towards the C-terminal ovine PrP residues 145 - 162) should include the epitope of SHA31. Therefore, a band of 17 kDa PK-resistant fragment was expected to be seen with SHA31, but it was not visulaised in my assay. My result suggest that there is some kind of epitope masking occurring due to conformational changes to the immobilised prion fragments preventing SHA31 antibody from binding and showing similar binding patterns to SAF70.

Previous research has also shown that producing a 17 kDa PK-resistant fragment was dictated by the conditions of digestion, i.e. the presence of specific detergents (0.1% SDS, 0.1% Triton X-100) in the $rPrP^{sen}$ buffer , which could occlude PK cleavage sites resulting in an apparent resistance to proteolytic digestion by fragments of certain molecular weight (Smirnovas *et al*., 2009). Since all rPrP-PMCA reactions were treated with the same buffer (that included 0.05% (wt/vol) SDS and 0.05% Triton X-100) and exposed to the same conditions of reaction, it is unlikely that buffer conditions contributed to the detection of PK $rPrP^{res(Sc)}$ or $rPrP^{res(spon)}$ within some replicates of the same seed. My data also argues against the possibility of contamination that might be the cause of producing these PK-resistant fragments. However, differences in tube positions have been found to influence intensity of amplification (Atarashi *et al*., 2007) although it is unlikely to explain the production of $rPrP^{res(spon)}$ in my study. Interestingly, decreasing the concentration of VRQ rPrP substrate to 0.1 mg/ml produced higher specificity as I did not detect any PK $rPrP^{res(spon)}$ from healthy seeded or non-seeded control samples. Therefore, under the rPrP-PMCA condition that I tested, my results suggest that the generation of both PK $rPrP^{res(Sc)}$ and PK $rPrP^{res\text{-}spon}$ depends on $rPrP^{sen}$ substrate sequence and concentration, variations between batches, together with the number of sPMCA rounds within each experiment. In addition, the variability observed between some replicate reactions using both substrates, suggest the importance of analyzing multiple replicates for accurate detection of PK $rPrP^{res(Sc)}$. Previous investigations into the variations within different batches of purified recombinant protein, found that despite a robust protocol for purification, batch to batch variation affected the morphology of amyloid fibril production (Ostapchenko *et al*., 2008). So, this observation might also explain the variation of amplification fidelity between different batches of the VRQ rPrP produced in my study. Furthermore, the amount of pure PrP^{Sc} in each seed and other co-factors contained within each PrP^{Sc} strain may influence the efficiency of amplification. Therefore, all these factors should be considered in order to establish a sensitive diagnostic assay with high accuracy.

Collectively, the present findings have important methodological implications. Since, the pattern of PK digestion has been used as a marker for the generation of PK-resistant fragments that confirm efficient amplification, more experimental optimisations coupled with mass spectrometry analysis are needed to differentiate between PK $rPrP^{res(Sc)}$ and PK $rPrP^{res(spon)}$, particularly when using VRQ rPrP

substrate. VRQ rPrP substrates have shown batch to batch variation that highlights the need to define each batch of substrate in terms of specificity before use. From my data, rSha PrP was a better substrate with improved specificity and sensitivity, as well as efficient amplification of all classical scrapie PrP^{Sc} seeded sPMCA reactions analysed. rSha PrP did not show batch to batch variation indicating that it is a more robust substrate for PrP^{Sc} amplification. rPrP has the advantage of being easily generated in high purity and in large amounts that could overcome the limitations of conventional sPMCA and facilitate the practicality of the technique as a diagnostic assay. Furthermore, comparing efficiency of amplification using brain homogenate as substrate (chapter: 5: *In Vitro* Amplification of Different PrP^{Sc} Strains in Substrates of Different Genotypes) with consideration to the differences in biological (brain tissue components) and chemical factors (detergents and proteases) included in each reaction, rPrP-PMCA was significantly more rapid than standard sPMCA. Importantly, the use of rSha PrP as a substrate for PMCA allows the detection of all classical scrapie isolates analysed irrespective of *Prnp* genotype. It also detected 3 out of 4 CH1641 samples. This represents a significant step forward in allowing the use of PMCA for the development of high sensitivity tests for scrapie in sheep.

Although, I intended to apply this assay to detect PrP^{Sc} in buccal swabs of infected sheep in the pre-clinical phase of the disease and compare the efficacy of this rPrP-PMCA assay with what has been described previously using brain homogenate as a substrate (Gough *et al*., 2012, Maddison *et al*., 2010), I was limited by the duration of this project. Further application of these assays to such biological samples is required to establish the use of rPrP-PMCA assay in the development of a live animal diagnostic for prion diseases in sheep.

Chapter 7: Conclusion and Future Work

This thesis presents new findings to add to our understanding of the biological properties of disease-related PrP and to assist in the development of new diagnostic techniques. Firstly, divergent physicochemical properties of PrP^{Sc} towards proteases and the existence of thermolysin-sensitive / PK-resistant PrP^{Sc}. Secondly, the novel use of sPMCA to distinguish experimental BSE from CH1641 in ovine samples and thirdly, efficient and accurate amplification of classical scrapie PrP^{Sc} using rSha PrP substrate.

The present study demonstrates an ELISA for PrP^{Sc} detection that utilises the protease TH. Others have reported that TH preserves disease-related PrP in a full length form and has the potential of detecting PK-sensitive PrP^{Sc} in rodents passaged scrapie samples. Through the developed ELISA, the isolation of full length PrP^{Sc} and clearing PrP^{C} with TH has overcome the problem of not fully digesting all healthy PrP using the standard 50 µg/ml PK. In addition, the developed ELISA is a helpful tool for quantifying the amount of full length PrP^{Sc} in samples in comparison to all PrP^{Sc} species including N-terminally truncated fragments (for example using Bio-Rad® TeSeE Sheep/Goat ELISA).

The study also describes TH-sensitive PK-resistant PrP^{Sc} that was detected from different ovine scrapie isolates. The presence of these isoforms varied between different brain regions of classical scrapie affected sheep and between individual sheep with the same prion infection. Previous studies hypothesised that different cell types process abnormal PrP in different ways and certain nerve cells might be more liable to endogenous proteolysis than others (Dron *et al*., 2010, Gonzalez *et al*., 2003). In turn, these tissue-specific differences may offer different environments for PrP^{Sc} selection within a single host and therefore result in dominant PrP^{Sc} conformers with different susceptibilities to exogenous proteases (Collinge and Clarke, 2007). Therefore, my finding also confirms the divergent physicochemical properties of PrP^{Sc} isoforms towards proteases and also reflects differences in the pathological process by different tissues and cell types for propagating PrP^{Sc} and producing heterogeneous populations of disease related PrP.

Other studies have reported TH-resistant / PK-sensitive PrP^{Sc} isoforms in rodents (Cronier *et al*., 2008) and existence of the PK-sensitive PrP^{Sc} isoform in sheep with scrapie (Thackray *et al*., 2007). Since PrP^{Sc} is the only marker for prion diseases

through its resistance to proteases, further work is recommended to examine the availability of PrP^{Sc} that displays differential protease susceptibility in different tissues, secreta and excreta of different species, together with using other proteases such as Pronase E. Moreover, analysing samples by CDI may enable the quantitation of the percentage of protease sensitive PrP^{Sc} in comparison of total PrP^{Sc} in an infected sample. This proposed study may improve diagnostic tests as proteases with improved diagnostic sensitivity for PrP^{Sc} might exist and recognise a large proportion of the PrP^{Sc} population.

The characteristaion of isolates of ovine BSE and CH1641 with the protease TH and PK also offered preliminary evidence that these strains may be distinguished by TH digestion followed by detection with the mAb P4. Experimental CH1641 presented PrP^{Sc} with two very distinct molecular phenotypes indicating divergent PrP^{Sc} conformer replication pathways in individuals infected with same prion strain. These data clearly indicate the complex nature of *in vivo* prion replication and the difficulties in defining prion strains at the molecular level.

Based on the protein hypothesis, generation of PrP^{Sc} *in vivo* occurs following direct interaction of PrP^{C} with the pathogenic protein, which acts as a template to drive the conformational change to PrP^{Sc}, and subsequently further conversion of the normal PrP^{C} molecule into PrP^{Sc}. This conversion process can be achieved *in vitro* using PMCA. Serial PMCA was initially tested on scrapie samples of susceptible genotype mainly homozygous VRQ PrP^{Sc} seeded homozygous VRQ substrate. Using diverse strains of ruminant prions and *Prnp* genotypes substrates in this study had considerable success for detecting minute amounts of PrP^{Sc} which is in agreement with the protein hypothesis. As the molecular weight of amplified products did not change from the molecular characteristics of the original seed upon *in vitro* amplification, it shows that conversion of PrP^{C} in PMCA reactions could reflect conversion of host PrP^{C} in *vivo* particularly in experimental passage of particular strain into different generations of particular species. It also demonstrated the complex nature of prion strains propagation. The data supports the influence of substrate and seed *Prnp* genotypes as well as scrapie strain on *in vitro* conversion efficiency. This further coincides with the role of the *Prnp* genetic code in determining susceptibility and resistance to prion diseases *in vivo*.

A novel result in my study is the successful application of sPMCA to the differentiation of ovine BSE and CH1641 prion strains based on their distinct *in vitro* replication properties. This initial success has since been validated in repeated experiments and in the diagnosis of CH1641-like field isolates (Thorne *et al*., unpublished data). Given the high sensitivity of sPMCA, these data also suggest the possibility of a promising diagnostic test to detect ovine BSE during preclinical or subclinical infections. At the same time, although CH1641 was refractory to amplification upon 5 rounds of PMCA reaction, the addition of poly(A) RNA to the PMCA reactions yielded positive amplification of this strain (Thorne *et al*., unpublished data). This indicates that cellular factors other than PrP^{C} might be required to stimulate efficient PrP^{Sc} production and host RNA molecules may have a role in the pathogenesis of prion diseases.

Taken all factors together, in order to use PMCA to screen for a wide range of field scrapie isolates, the use of multiple *Prnp* genotypes, as well as non-PrP factors may be required to achieve optimal amplification efficiency.

Using rPrP as a substrate for sPMCA has overcome most of the limitations of using brain homogenate as substrate in the standard sPMCA. The main advantages are a continuous supply of rPrP and rapid detection of PrP^{Sc} with accurate specificity of classical scrapie regardless of strain or host genotype.

Comparing the sensitivity of sPMCA and rPrP-PMCA, both rSha PrP and VRQ rPrP supported amplification of all classical scrapie and CH1641 isolates, independent of seed *Prnp* genotype. These amplification efficiencies were not obtained using different ovine *Prnp* brain homogenate substrates. Furthermore, variable amplification results were obtained in rPrP-PMCA reaction for ovine BSE samples. It seems that using brain homogenate as a source of PrP^{C} could be more sensitive than using rPrP for particular strains like ovine BSE and SSBP1. This might be related to the contents of proteins, lipids, glycosylphosphatidylinsitol (GPI) anchor and nucleic acids within brain homogenate substrate that support replication of these particular strains and sustained conversion of PrP^{C}. On the other hand, although the specificity of rPrP VRQ varied between batches, efficiency of amplification was found between ovine brain homogenates of similar genotypes (B. Maddison, personal communication). In addition, recent study of comparing amplification efficiency for particular human prion diseases like vCJD and sCJD using rPrP as a

substrate showed that there are mechanistic differences between amplification techniques like PMCA and QuIC that could affect accumulation of PrP^{Sc} *in vitro* (Peden *et al*., 2012).

Therefore, my study suggest that *in vitro* amplification efficiencies of TSEs in sheep could be driven by the nature of the seeds, substrates and the biochemical condition of PMCA reaction. Moreover, rPrP-PMCA may provide convenient and robust assay for ovine scrapie strains amplification (i.e. classical scrapie and CH1641 strains) but not for ovine BSE. Further applications and comparison of both sPMCA and rPrP-PMCA to detect PrP^{Sc} in secreta and excreta of infected sheep in the pre-clinical phase of the disease is of high importance to validate the practicality of the PMCA technique as a future diagnostic assay to field cases of a wide range of strains and genotypes. Moreover, further experiments are required to examine the difference of amplification techniques' mechanism and comparison between PMCA and QuIC in relation to amplification of sheep TSE strains *in vitro*.

References:

AGUZZI, A., BAUMANN, F. & BREMER, J. 2008. The prion's elusive reason for being. *Annu Rev Neurosci,* 31**,** 439-77.

ALDHOUS, P. 1990. BSE: spongiform encephalopathy found in cat. *Nature,* 345**,** 194.

ANDREOLETTI, O., BERTHON, P., MARC, D., SARRADIN, P., GROSCLAUDE, J., VAN KEULEN, L., SCHELCHER, F., ELSEN, J. M. & LANTIER, F. 2000. 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,* 81**,** 3115-26.

ANDREOLETTI, O., LACROUX, C., CHABERT, A., MONNEREAU, L., TABOURET, G., LANTIER, F., BERTHON, P., EYCHENNE, F., LAFOND-BENESTAD, S., ELSEN, J. M. & SCHELCHER, F. 2002. PrP(Sc) accumulation in placentas of ewes exposed to natural scrapie: influence of foetal PrP genotype and effect on ewe-to-lamb transmission. *J Gen Virol,* 83**,** 2607-16.

ANGERS, R. C., SEWARD, T. S., NAPIER, D., GREEN, M., HOOVER, E., SPRAKER, T., O'ROURKE, K., BALACHANDRAN, A. & TELLING, G. C. 2009. Chronic wasting disease prions in elk antler velvet. *Emerg Infect Dis,* 15**,** 696-703.

ATARASHI, R., MOORE, R. A., SIM, V. L., HUGHSON, A. G., DORWARD, D. W., ONWUBIKO, H. A., PRIOLA, S. A. & CAUGHEY, B. 2007. Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. *Nat Methods,* 4**,** 645-50.

ATARASHI, R., SANO, K., SATOH, K. & NISHIDA, N. 2011[(b)]. Real-time quaking-induced conversion: a highly sensitive assay for prion detection. *Prion,* 5**,** 150-3.

ATARASHI, R., SATOH, K., SANO, K., FUSE, T., YAMAGUCHI, N., ISHIBASHI, D., MATSUBARA, T., NAKAGAKI, T., YAMANAKA, H., SHIRABE, S., YAMADA, M., MIZUSAWA, H., KITAMOTO, T., KLUG, G., MCGLADE, A., COLLINS, S. J. & NISHIDA, N. 2011[(a).] Ultrasensitive

human prion detection in cerebrospinal fluid by real-time quaking-induced conversion. *Nat Med,* 17**,** 175-8.

ATARASHI, R., SIM, V. L., NISHIDA, N., CAUGHEY, B. & KATAMINE, S. 2006. Prion strain-dependent differences in conversion of mutant prion proteins in cell culture. *J Virol,* 80**,** 7854-62.

ATARASHI, R., WILHAM, J. M., CHRISTENSEN, L., HUGHSON, A. G., MOORE, R. A., JOHNSON, L. M., ONWUBIKO, H. A., PRIOLA, S. A. & CAUGHEY, B. 2008. Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. *Nat Methods,* 5**,** 211-2.

BANNACH, O., BIRKMANN, E., REINARTZ, E., JAEGER, K. E., LANGEVELD, J. P., ROHWER, R. G., GREGORI, L., TERRY, L. A., WILLBOLD, D. & RIESNER, D. 2012. Detection of prion protein particles in blood plasma of scrapie infected sheep. *PLoS One,* 7**,** e36620.

BARNARD, G., HELMICK, B., MADDEN, S., GILBOURNE, C. & PATEL, R. 2000. The measurement of prion protein in bovine brain tissue using differential extraction and DELFIA as a diagnostic test for BSE. *Luminescence,* 15**,** 357-62.

BARON, T. & BIACABE, A. G. 2006. Origin of bovine spongiform encephalopathy. *Lancet,* 367**,** 297-8; author reply 298-9.

BARON, T. & BIACABE, A. G. 2007. Molecular behaviors of "CH1641-like" sheep scrapie isolates in ovine transgenic mice (TgOvPrP4). *J Virol,* 81**,** 7230-7.

BARON, T. G., MADEC, J. Y. & CALAVAS, D. 1999. Similar signature of the prion protein in natural sheep scrapie and bovine spongiform encephalopathy-linked diseases. *J Clin Microbiol,* 37**,** 3701-4.

BARON, T. G., MADEC, J. Y., CALAVAS, D., RICHARD, Y. & BARILLET, F. 2000. Comparison of French natural scrapie isolates with bovine spongiform encephalopathy and experimental scrapie infected sheep. *Neurosci Lett,* 284**,** 175-8.

BARON, T., BELLI, P., MADEC, J. Y., MOUTOU, F., VITAUD, C. & SAVEY, M. 1997. Spongiform encephalopathy in an imported cheetah in France. *Vet Rec,* 141**,** 270-1.

BARON, T., BENCSIK, A., VULIN, J., BIACABE, A. G., MORIGNAT, E., VERCHERE, J. & BETEMPS, D. 2008. A C-terminal protease-resistant prion fragment distinguishes ovine "CH1641-like" scrapie from bovine

classical and L-Type BSE in ovine transgenic mice. *PLoS Pathog,* 4**,** e1000137.

BASKAKOV, I. V. 2004. Autocatalytic conversion of recombinant prion proteins displays a species barrier. *J Biol Chem,* 279**,** 7671-7.

BASKAKOV, I. V. 2009. Switching in amyloid structure within individual fibrils: implication for strain adaptation, species barrier and strain classification. *FEBS Lett,* 583**,** 2618-22.

BASKAKOV, I. V., LEGNAME, G., BALDWIN, M. A., PRUSINER, S. B. & COHEN, F. E. 2002. Pathway complexity of prion protein assembly into amyloid. *J Biol Chem,* 277**,** 21140-8.

BATE, C., LANGEVELD, J. & WILLIAMS, A. 2004. Manipulation of PrPres production in scrapie-infected neuroblastoma cells. *J Neurosci Methods,* 138**,** 217-23.

BAYLIS, M. & GOLDMANN, W. 2004. The genetics of scrapie in sheep and goats. *Curr Mol Med,* 4**,** 385-96.

BECK, K. E., CHAPLIN, M., STACK, M., SALLIS, R. E., SIMONINI, S., LOCKEY, R. & SPIROPOULOS, J. 2010. Lesion profiling at primary isolation in RIII mice is insufficient in distinguishing BSE from classical scrapie. *Brain Pathol,* 20**,** 313-22.

BEEKES, M. & MCBRIDE, P. A. 2007. The spread of prions through the body in naturally acquired transmissible spongiform encephalopathies. *FEBS J,* 274**,** 588-605.

BEGARA-MCGORUM, I., GONZALEZ, L., SIMMONS, M., HUNTER, N., HOUSTON, F. & JEFFREY, M. 2002. Vacuolar lesion profile in sheep scrapie: factors influencing its variation and relationship to disease-specific PrP accumulation. *J Comp Pathol,* 127**,** 59-68.

BELLON, A., SEYFERT-BRANDT, W., LANG, W., BARON, H., GRONER, A. & VEY, M. 2003. Improved conformation-dependent immunoassay: suitability for human prion detection with enhanced sensitivity. *J Gen Virol,* 84**,** 1921-5.

BELLWORTHY, S. J., DEXTER, G., STACK, M., CHAPLIN, M., HAWKINS, S. A., SIMMONS, M. M., JEFFREY, M., MARTIN, S., GONZALEZ, L. & HILL, P. 2005. Natural transmission of BSE between sheep within an experimental flock. *Vet Rec,* 157**,** 206.

BELT, P. B., MUILEMAN, I. H., SCHREUDER, B. E., BOS-DE RUIJTER, J., GIELKENS, A. L. & SMITS, M. A. 1995. Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *J Gen Virol,* 76 (Pt 3)**,** 509-17.

BENDHEIM, P. E., BROWN, H. R., RUDELLI, R. D., SCALA, L. J., GOLLER, N. L., WEN, G. Y., KASCSAK, R. J., CASHMAN, N. R. & BOLTON, D. C. 1992. Nearly ubiquitous tissue distribution of the scrapie agent precursor protein. *Neurology,* 42**,** 149-56.

BENESTAD, S. L., SARRADIN, P., THU, B., SCHONHEIT, J., TRANULIS, M. A. & BRATBERG, B. 2003. Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Vet Rec,* 153**,** 202-8.

BÉRINGUE, V., VILOTTE, J. L., LAUDE, H. 2008. Prion agent diversity and species barrier. *Vet Res*., 39, 4:47-77

BESSEN, R. A., SHEARIN, H., MARTINKA, S., BOHARSKI, R., LOWE, D., WILHAM, J. M., CAUGHEY, B. & WILEY, J. A. 2010. Prion shedding from olfactory neurons into nasal secretions. *PLoS Pathog,* 6**,** e1000837.

BIACABE, A. G., LAPLANCHE, J. L., RYDER, S. & BARON, T. 2004. Distinct molecular phenotypes in bovine prion diseases. *EMBO Rep,* 5**,** 110-5.

BILHEUDE, J. M., BRUN, A., MOREL, N., DIAZ SAN SEGUNDO, F., LECROIX, S., ESPINOSA, J. C., GONZALEZ, L., STEELE, P., GRASSI, J., ANDREOLETTI, O. & TORRES, J. M. 2007. Discrimination of sheep susceptible and resistant to transmissible spongiform encephalopathies by an haplotype specific monoclonal antibody. *J Virol Methods,* 145**,** 169-72.

BILLINIS, C., PSYCHAS, V., LEONTIDES, L., SPYROU, V., ARGYROUDIS, S., VLEMMAS, I., LEONTIDES, S., SKLAVIADIS, T. & PAPADOPOULOS, O. 2004. Prion protein gene polymorphisms in healthy and scrapie-affected sheep in Greece. *J Gen Virol,* 85**,** 547-54.

BOCHAROVA, O. V., BREYDO, L., SALNIKOV, V. V., GILL, A. C. & BASKAKOV, I. V. 2005. Synthetic prions generated in vitro are similar to a newly identified subpopulation of PrPSc from sporadic Creutzfeldt-Jakob Disease. *Protein Sci,* 14**,** 1222-32.

BONS, N., MESTRE-FRANCES, N., BELLI, P., CATHALA, F., GAJDUSEK, D. C. & BROWN, P. 1999. Natural and experimental oral infection of nonhuman

primates by bovine spongiform encephalopathy agents. *Proc Natl Acad Sci U S A,* 96**,** 4046-51.

BOSSERS, A., BELT, P., RAYMOND, G. J., CAUGHEY, B., DE VRIES, R. & SMITS, M. A. 1997. Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms. *Proc Natl Acad Sci U S A,* 94**,** 4931-6.

BOSSERS, A., DE VRIES, R. & SMITS, M. A. 2000. Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. *J Virol,* 74**,** 1407-14.

BROWN, D. R., NICHOLAS, R. S. & CANEVARI, L. 2002. Lack of prion protein expression results in a neuronal phenotype sensitive to stress. *J Neurosci Res,* 67**,** 211-24.

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. 1997. The cellular prion protein binds copper in vivo. *Nature,* 390**,** 684-7.

BROWN, P., WILL, R. G., BRADLEY, R., ASHER, D. M. & DETWILER, L. 2001. Bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease: background, evolution, and current concerns. *Emerg Infect Dis,* 7**,** 6-16.

BRUCE, M. E. & FRASER, H. 1991. Scrapie strain variation and its implications. *Curr Top Microbiol Immunol,* 172**,** 125-38.

BRUCE, M. E. 1993. Scrapie strain variation and mutation. *Br Med Bull,* 49**,** 822-38.

BRUCE, M. E. 2003. TSE strain variation. *Br Med Bull,* 66**,** 99-108.

BRUCE, M. E., BOYLE, A., COUSENS, S., MCCONNELL, I., FOSTER, J., GOLDMANN, W. & FRASER, H. 2002. Strain characterization of natural sheep scrapie and comparison with BSE. *J Gen Virol,* 83**,** 695-704.

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. 1997. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature,* 389**,** 498-501.

BUCALOSSI, C., COSSEDDU, G., D'AGOSTINO, C., DI BARI, M. A., CHIAPPINI, B., CONTE, M., ROSONE, F., DE GROSSI, L., SCAVIA, G., AGRIMI, U., NONNO, R. & VACCARI, G. 2011. Assessment of the genetic

susceptibility of sheep to scrapie by protein misfolding cyclic amplification and comparison with experimental scrapie transmission studies. *J Virol,* 85**,** 8386-92.

BUELER, H., FISCHER, M., LANG, Y., BLUETHMANN, H., LIPP, H. P., DEARMOND, S. J., PRUSINER, S. B., AGUET, M. & WEISSMANN, C. 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature,* 356**,** 577-82.

BUSCHMANN, A., GRETZSCHEL, A., BIACABE, A. G., SCHIEBEL, K., CORONA, C., HOFFMANN, C., EIDEN, M., BARON, T., CASALONE, C. & GROSCHUP, M. H. 2006. Atypical BSE in Germany--proof of transmissibility and biochemical characterization. *Vet Microbiol,* 117**,** 103-16.

BUSCHMANN, A., LUHKEN, G., SCHULTZ, J., ERHARDT, G. & GROSCHUP, M. H. 2004. Neuronal accumulation of abnormal prion protein in sheep carrying a scrapie-resistant genotype (PrPARR/ARR). *J Gen Virol,* 85**,** 2727-33.

CANCELLOTTI, E., BARRON, R. M., BISHOP, M. T., HART, P., WISEMAN, F. & MANSON, J. C. 2007. The role of host PrP in Transmissible Spongiform Encephalopathies. *Biochim Biophys Acta,* 1772**,** 673-80.

CASALONE, C., ZANUSSO, G., ACUTIS, P., FERRARI, S., CAPUCCI, L., TAGLIAVINI, F., MONACO, S. & CARAMELLI, M. 2004. Identification of a second bovine amyloidotic spongiform encephalopathy: molecular similarities with sporadic Creutzfeldt-Jakob disease. *Proc Natl Acad Sci U S A,* 101**,** 3065-70.

CASTILLA, J., SAA, P. & SOTO, C. 2005b. Detection of prions in blood. *Nat Med,* 11**,** 982-5.

CASTILLA, J., SAA, P., HETZ, C. & SOTO, C. 2005a. In vitro generation of infectious scrapie prions. *Cell,* 121**,** 195-206.

CASTILLA, J., SAA, P., MORALES, R., ABID, K., MAUNDRELL, K. & SOTO, C. 2006. Protein misfolding cyclic amplification for diagnosis and prion propagation studies. *Methods Enzymol,* 412**,** 3-21

CAUGHEY, B., KOCISKO, D. A., RAYMOND, G. J. & LANSBURY, P. T., JR. 1995. Aggregates of scrapie-associated prion protein induce the cell-free

conversion of protease-sensitive prion protein to the protease-resistant state. *Chem Biol,* 2**,** 807-17.

CHANG, B., CHENG, X., YIN, S., PAN, T., ZHANG, H., WONG, P., KANG, S. C., XIAO, F., YAN, H., LI, C., WOLFE, L. L., MILLER, M. W., WISNIEWSKI, T., GREENE, M. I. & SY, M. S. 2007. Test for detection of disease-associated prion aggregate in the blood of infected but asymptomatic animals. *Clin Vaccine Immunol,* 14**,** 36-43.

CHEN, S., MANGE, A., DONG, L., LEHMANN, S., SCHACHNER, M. 2003. Prion protein as trans-interacting partner for neurons is involved in neurite outgrowth and neuronal survival. *Mol Cell Neurosci,* 22:227–233.

CHESEBRO, B., TRIFILO, M., RACE, R., MEADE-WHITE, K., TENG, C., LACASSE, R., RAYMOND, L., FAVARA, C., BARON, G., PRIOLA, S., CAUGHEY, B., MASLIAH, E. & OLDSTONE, M. 2005. Anchorless prion protein results in infectious amyloid disease without clinical scrapie. *Science,* 308**,** 1435-9.

CLOUSCARD, C., BEAUDRY, P., ELSEN, J. M., MILAN, D., DUSSAUCY, M., BOUNNEAU, C., SCHELCHER, F., CHATELAIN, J., LAUNAY, J. M. & LAPLANCHE, J. L. 1995. Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie. *J Gen Virol,* 76 (Pt 8)**,** 2097-101.

COLLINGE, J. & CLARKE, A. R. 2007. A general model of prion strains and their pathogenicity. *Science,* 318**,** 930-6.

CORTELLI, P., GAMBETTI, P., MONTAGNA, P. & LUGARESI, E. 1999. Fatal familial insomnia: clinical features and molecular genetics. *J Sleep Res,* 8 Suppl 1**,** 23-9.

CREMINON, C., GRASSI, J. Characterization of anti-PrP antibodies and measurement of PrP using ELISA techniques. In: LEHMANN, S., GRASSI, J. (2004) Techniques in Prion Research, eds. 117-31, Birkhauser Verlag Basel / Switzerland

CRONIER, S., GROS, N., TATTUM, M. H., JACKSON, G. S., CLARKE, A. R., COLLINGE, J. & WADSWORTH, J. D. 2008. Detection and characterization of proteinase K-sensitive disease-related prion protein with thermolysin. *Biochem J,* 416**,** 297-305.

CUNNINGHAM, A. A., WELLS, G. A., SCOTT, A. C., KIRKWOOD, J. K. & BARNETT, J. E. 1993. Transmissible spongiform encephalopathy in greater kudu (Tragelaphus strepsiceros). *Vet Rec,* 132**,** 68.

DAVIES, D. C. & KIMBERLIN, R. H. 1985. Selection of Swaledale sheep of reduced susceptibility to experimental scrapie. *Vet Rec,* 116**,** 211-4.

DELEAULT, N. R., GEOGHEGAN, J. C., NISHINA, K., KASCSAK, R., WILLIAMSON, R. A. & SUPATTAPONE, S. 2005. Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. *J Biol Chem*, 280, 26873–26879.

DELEAULT, N. R., HARRIS, B. T., REES, J. R. & SUPATTAPONE, S. 2007. Formation of native prions from minimal components in vitro. *Proc Natl Acad Sci*, 104, 9741–9746.

DELEAULT, N. R., LUCASSEN, R. W. & SUPATTAPONE, S. 2003. RNA molecules stimulate prion protein conversion. *Nature,* 425, 717–720.

DESLY, J. P., PICOT, A., (2002): MAD COW DISEASE: The Risks to Humans. Flammarion 2002 eds, for the English translation, Hérissey, Evreux, France.

DICKINSON, A. G. 1976. Scrapie in sheep and goats. *Front Biol,* 44**,** 209-41.

DICKINSON, A. G., MEIKLE, V. M. & FRASER, H. 1968. Identification of a gene which controls the incubation period of some strains of scrapie agent in mice. *J Comp Pathol,* 78**,** 293-9.

DOHERR, M. G. 2007. Brief review on the epidemiology of transmissible spongiform encephalopathies (TSE). *Vaccine,* 25**,** 5619-24.

DRON, M., MOUDJOU, M., CHAPUIS, J., SALAMAT, M. K., BERNARD, J., CRONIER, S., LANGEVIN, C. & LAUDE, H. 2010. Endogenous proteolytic cleavage of disease-associated prion protein to produce C2 fragments is strongly cell- and tissue-dependent. *J Biol Chem,* 285**,** 10252-64.

DUFFY, P., WOLF, J., COLLINS, G., DEVOE, A. G., STREETEN, B. & COWEN, D. 1974. Letter: Possible person-to-person transmission of Creutzfeldt-Jakob disease. *N Engl J Med,* 290**,** 692-3.

EIDEN, M., PALM, G. J., HINRICHS, W., MATTHEY, U., ZAHN, R. & GROSCHUP, M. H. 2006. Synergistic and strain-specific effects of bovine spongiform encephalopathy and scrapie prions in the cell-free conversion of recombinant prion protein. *J Gen Virol,* 87**,** 3753-61.

ELOIT, M., ADJOU, K., COULPIER, M., FONTAINE, J. J., HAMEL, R., LILIN, T., MESSIAEN, S., ANDREOLETTI, O., BARON, T., BENCSIK, A., BIACABE, A. G., BERINGUE, V., LAUDE, H., LE DUR, A., VILOTTE, J. L., COMOY, E., DESLYS, J. P., GRASSI, J., SIMON, S., LANTIER, F. & SARRADIN, P. 2005. BSE agent signatures in a goat. *Vet Rec,* 156**,** 523-4.

EUROSURVEILLANCE, EDITORIAL TEAM (2005). BSE agent in goat tissue: first known naturally occurring case confirmed. Euro Surveill. 2005;10(5):pii=2633.Availableonline: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=2633

EVEREST, S. J., RAMSAY, A. M., CHAPLIN, M. J., EVERITT, S., STACK, M. J., NEALE, M. H., JEFFREY, M., MOORE, S. J., BELLWORTHY, S. J. & TERRY, L. A. 2011. Detection and localisation of PrP(Sc) in the liver of sheep infected with scrapie and bovine spongiform encephalopathy. *PLoS One,* 6**,** e19737.

FERAUDET, C., MOREL, N., SIMON, S., VOLLAND, H., FROBERT, Y., CREMINON, C., VILETTE, D., LEHMANN, S. & GRASSI, J. 2005. Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. *J Biol Chem,* 280**,** 11247-58.

FOSTER, J. D. & DICKINSON, A. G. 1988. The unusual properties of CH1641, a sheep-passaged isolate of scrapie. *Vet Rec,* 123**,** 5-8.

FRASER, H. 1976. The pathology of a natural and experimental scrapie. *Front Biol,* 44**,** 267-305.

GAJDUSEK, D. C. & ZIGAS, V. 1957. Degenerative disease of the central nervous system in New Guinea; the endemic occurrence of kuru in the native population. *N Engl J Med,* 257**,** 974-8.

GAJDUSEK, D. C. 1988. Transmissible and non-transmissible amyloidoses: autocatalytic post-translational conversion of host precursor proteins to beta-pleated sheet configurations. *J Neuroimmunol,* 20**,** 95-110.

GAVIER-WIDEN, D., NOREMARK, M., LANGEVELD, J. P., STACK, M., BIACABE, A. G., VULIN, J., CHAPLIN, M., RICHT, J. A., JACOBS, J., ACIN, C., MONLEON, E., RENSTROM, L., KLINGEBORN, B. & BARON, T. G. 2008. Bovine spongiform encephalopathy in Sweden: an H-type variant. *J Vet Diagn Invest,* 20**,** 2-10.

GAVIER-WIDEN, D., STACK, M. J., BARON, T., BALACHANDRAN, A. & SIMMONS, M. 2005. Diagnosis of transmissible spongiform encephalopathies in animals: a review. *J Vet Diagn Invest,* 17**,** 509-27.

GHAEMMAGHAMI, S., PHUAN, P. W., PERKINS, B., ULLMAN, J., MAY, B. C., COHEN, F. E. & PRUSINER, S. B. 2007. Cell division modulates prion accumulation in cultured cells. *Proc Natl Acad Sci U S A,* 104**,** 17971-6.

GILCH, S., CHITOOR, N., TAGUCHI, Y., STUART, M., JEWELL, J. E. & SCHATZL, H. M. 2011. Chronic wasting disease. *Top Curr Chem,* 305**,** 51-77.

GOLDFARB, L. G., PETERSEN, R. B., TABATON, M., BROWN, P., LEBLANC, A. C., MONTAGNA, P., CORTELLI, P., JULIEN, J., VITAL, C., PENDELBURY, W. W. & ET AL. 1992. Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism. *Science,* 258**,** 806-8.

GOLDMANN, W. 2008. PrP genetics in ruminant transmissible spongiform encephalopathies. *Vet Res,* 39**,** 30.

GOLDMANN, W., HUNTER, N., BENSON, G., FOSTER, J. D. & HOPE, J. 1991. Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the Sip gene. *J Gen Virol,* 72 (Pt 10)**,** 2411-7.

GOLDMANN, W., HUNTER, N., SMITH, G., FOSTER, J. & HOPE, J. 1994. PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. *J Gen Virol,* 75 (Pt 5)**,** 989-95.

GONZALEZ, L., DAGLEISH, M. P., MARTIN, S., DEXTER, G., STEELE, P., FINLAYSON, J. & JEFFREY, M. 2008a. Diagnosis of preclinical scrapie in live sheep by the immunohistochemical examination of rectal biopsies. *Vet Rec,* 162**,** 397-403.

GONZALEZ, L., HORTON, R., RAMSAY, D., TOOMIK, R., LEATHERS, V., TONELLI, Q., DAGLEISH, M. P., JEFFREY, M. & TERRY, L. 2008b. Adaptation and evaluation of a rapid test for the diagnosis of sheep scrapie in samples of rectal mucosa. *J Vet Diagn Invest,* 20**,** 203-8.

GONZALEZ, L., MARTIN, S. & JEFFREY, M. 2003. Distinct profiles of PrP(d) immunoreactivity in the brain of scrapie- and BSE-infected sheep:

implications for differential cell targeting and PrP processing. *J Gen Virol,* 84**,** 1339-50.

GONZALEZ, L., MARTIN, S., BEGARA-MCGORUM, I., HUNTER, N., HOUSTON, F., SIMMONS, M. & JEFFREY, M. 2002. Effects of agent strain and host genotype on PrP accumulation in the brain of sheep naturally and experimentally affected with scrapie. *J Comp Pathol,* 126**,** 17-29.

GONZALEZ, L., MARTIN, S., HOUSTON, F. E., HUNTER, N., REID, H. W., BELLWORTHY, S. J. & JEFFREY, M. 2005. Phenotype of disease-associated PrP accumulation in the brain of bovine spongiform encephalopathy experimentally infected sheep. *J Gen Virol,* 86**,** 827-38.

GONZALEZ, L., SISO, S., MONLEON, E., CASALONE, C., VAN KEULEN, L. J., BALKEMA-BUSCHMANN, A., ORTIZ-PELAEZ, A., IULINI, B., LANGEVELD, J. P., HOFFMANN, C., BADIOLA, J. J., JEFFREY, M. & ACIN, C. 2010. Variability in disease phenotypes within a single PRNP genotype suggests the existence of multiple natural sheep scrapie strains within Europe. *J Gen Virol,* 91**,** 2630-41.

GONZALEZ-ROMERO, D., BARRIA, M. A., LEON, P., MORALES, R. & SOTO, C. 2008. Detection of infectious prions in urine. *FEBS Lett,* 582**,** 3161-6.

GOSSNER, A. G., BENNET, N., HUNTER, N. & HOPKINS, J. 2009. Differential expression of Prnp and Sprn in scrapie infected sheep also reveals Prnp genotype specific differences. *Biochem Biophys Res Commun,* 378**,** 862-6.

GOUGH, K. C. & MADDISON, B. C. 2010. Prion transmission: prion excretion and occurrence in the environment. *Prion,* 4**,** 275-82.

GOUGH, K. C., BAKER, C. A., REES, H. C., TERRY, L. A., SPIROPOULOS, J., THORNE, L. & MADDISON, B. C. 2012. The oral secretion of infectious scrapie prions occurs in preclinical sheep with a range of PRNP genotypes. *J Virol,* 86**,** 566-71.

GOUGH, K. C., BAKER, C. A., TAEMA, M. & MADDISON, B. C. 2009. In vitro amplification of prions from milk in the detection of subclinical infections. *Prion,* 3**,** 236-9.

GRASSI, J., MAILLET, S., SIMON, S. & MOREL, N. 2008. Progress and limits of TSE diagnostic tools. *Vet Res,* 39**,** 33.

GREGORI, L., KOVACS, G. G., ALEXEEVA, I., BUDKA, H. & ROHWER, R. G. 2008. Excretion of transmissible spongiform encephalopathy infectivity in urine. *Emerg Infect Dis,* 14**,** 1406-12.

GRETZSCHEL, A., BUSCHMANN, A., LANGEVELD, J. & GROSCHUP, M. H. 2006. Immunological characterization of abnormal prion protein from atypical scrapie cases in sheep using a panel of monoclonal antibodies. *J Gen Virol,* 87**,** 3715-22.

HADLOW, W. J. & KARSTAD, L. 1968. Transmissible encephalopathy of mink in Ontario. *Can Vet J,* 9**,** 193-6.

HADLOW, W. J. 1959. Scrapie and Kuru. *Lancet,* 2**,** 289-290.

HALEY, N. J., SEELIG, D. M., ZABEL, M. D., TELLING, G. C. & HOOVER, E. A. 2009. Detection of CWD prions in urine and saliva of deer by transgenic mouse bioassay. *PLoS One,* 4**,** e4848.

HAMIR, A. N., KUNKLE, R. A., CUTLIP, R. C., MILLER, J. M., WILLIAMS, E. S. & RICHT, J. A. 2006. Transmission of chronic wasting disease of mule deer to Suffolk sheep following intracerebral inoculation. *J Vet Diagn Invest,* 18**,** 558-65.

HAMIR, A. N., KUNKLE, R. A., MILLER, J. M., GREENLEE, J. J. & RICHT, J. A. 2006. Experimental second passage of chronic wasting disease (CWD(mule deer)) agent to cattle. *J Comp Pathol,* 134**,** 63-9.

HAMIR, A. N., RICHT, J. A., MILLER, J. M., KUNKLE, R. A., HALL, S. M., NICHOLSON, E. M., O'ROURKE, K. I., GREENLEE, J. J. & WILLIAMS, E. S. 2008. Experimental transmission of chronic wasting disease (CWD) of elk (Cervus elaphus nelsoni), white-tailed deer (Odocoileus virginianus), and mule deer (Odocoileus hemionus hemionus) to white-tailed deer by intracerebral route. *Vet Pathol,* 45**,** 297-306.

HARMEYER, S., PFAFF, E. & GROSCHUP, M. H. 1998. Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. *J Gen Virol,* 79 (Pt 4)**,** 937-45.

HEGGEBO, R., PRESS, C. M., GUNNES, G., LIE, K. I., TRANULIS, M. A., ULVUND, M., GROSCHUP, M. H. & LANDSVERK, T. 2000. Distribution of prion protein in the ileal Peyer's patch of scrapie-free lambs and lambs naturally and experimentally exposed to the scrapie agent. *J Gen Virol,* 81**,** 2327-37.

HEISEY, D. M., MICKELSEN, N. A., SCHNEIDER, J. R., JOHNSON, C. J., JOHNSON, C. J., LANGENBERG, J. A., BOCHSLER, P. N., KEANE, D. P. & BARR, D. J. 2010. Chronic wasting disease (CWD) susceptibility of several North American rodents that are sympatric with cervid CWD epidemics. *J Virol,* 84**,** 210-5.

HILL, A. F., DESBRUSLAIS, M., JOINER, S., SIDLE, K. C., GOWLAND, I., COLLINGE, J., DOEY, L. J. & LANTOS, P. 1997. The same prion strain causes vCJD and BSE. *Nature,* 389**,** 448-50, 526.

HILL, A. F., JOINER, S., LINEHAN, J., DESBRUSLAIS, M., LANTOS, P. L. & COLLINGE, J. 2000. Species-barrier-independent prion replication in apparently resistant species. *Proc Natl Acad Sci U S A,* 97**,** 10248-53.

HILL, A. F., SIDLE, K. C., JOINER, S., KEYES, P., MARTIN, T. C., DAWSON, M. & COLLINGE, J. 1998. Molecular screening of sheep for bovine spongiform encephalopathy. *Neurosci Lett,* 255**,** 159-62.

HOPE, J., WOOD, S. C., BIRKETT, C. R., CHONG, A., BRUCE, M. E., CAIRNS, D., GOLDMANN, W., HUNTER, N. & BOSTOCK, C. J. 1999. Molecular analysis of ovine prion protein identifies similarities between BSE and an experimental isolate of natural scrapie, CH1641. *J Gen Virol,* 80 (Pt 1)**,** 1-4.

HORIUCHI, M. & CAUGHEY, B. 1999. Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state. *EMBO J,* 18**,** 3193-203.

HORIUCHI, M., PRIOLA, S. A., CHABRY, J. & CAUGHEY, B. 2000. Interactions between heterologous forms of prion protein: binding, inhibition of conversion, and species barriers. *Proc Natl Acad Sci U S A,* 97**,** 5836-41.

HOUSTON, E. F., HALLIDAY, S. I., JEFFREY, M., GOLDMANN, W. & HUNTER, N. 2002. New Zealand sheep with scrapie-susceptible PrP genotypes succumb to experimental challenge with a sheep-passaged scrapie isolate (SSBP/1). *J Gen Virol,* 83**,** 1247-50.

HUNTER, N. 2003. Scrapie and experimental BSE in sheep. *Br Med Bull,* 66**,** 171-83.

HUNTER, N. 2007. Scrapie: uncertainties, biology and molecular approaches. *Biochim Biophys Acta,* 1772**,** 619-28.

HUNTER, N., FOSTER, J. D., GOLDMANN, W., STEAR, M. J., HOPE, J. & BOSTOCK, C. 1996. Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Arch Virol,* 141**,** 809-24.

HUNTER, N., GOLDMANN, W., BENSON, G., FOSTER, J. D. & HOPE, J. 1993. Swaledale sheep affected by natural scrapie differ significantly in PrP genotype frequencies from healthy sheep and those selected for reduced incidence of scrapie. *J Gen Virol,* 74 (Pt 6)**,** 1025-31.

IMRAN, M. & MAHMOOD, S. 2011. An overview of animal prion diseases. *Virol J,* 8**,** 493.

JACKMAN, R., EVEREST, D. J., SCHMERR, M. J., KHAWAJA, M., KEEP, P. & DOCHERTY, J. 2006. Evaluation of a preclinical blood test for scrapie in sheep using immunocapillary electrophoresis. *J AOAC Int,* 89**,** 720-7.

JACOBS, J. G., BOSSERS, A., REZAEI, H., VAN KEULEN, L. J., MCCUTCHEON, S., SKLAVIADIS, T., LANTIER, I., BERTHON, P., LANTIER, F., VAN ZIJDERVELD, F. G. & LANGEVELD, J. P. 2011. Proteinase K-resistant material in ARR/VRQ sheep brain affected with classical scrapie is composed mainly of VRQ prion protein. *J Virol,* 85**,** 12537-46.

JACOBS, J. G., LANGEVELD, J. P., BIACABE, A. G., ACUTIS, P. L., POLAK, M. P., GAVIER-WIDEN, D., BUSCHMANN, A., CARAMELLI, M., CASALONE, C., MAZZA, M., GROSCHUP, M., ERKENS, J. H., DAVIDSE, A., VAN ZIJDERVELD, F. G. & BARON, T. 2007. Molecular discrimination of atypical bovine spongiform encephalopathy strains from a geographical region spanning a wide area in Europe. *J Clin Microbiol,* 45**,** 1821-9.

JACOBS, J. G., SAUER, M., VAN KEULEN, L. J., TANG, Y., BOSSERS, A. & LANGEVELD, J. P. 2011. Differentiation of ruminant transmissible spongiform encephalopathy isolate types, including bovine spongiform encephalopathy and CH1641 scrapie. *J Gen Virol,* 92**,** 222-32.

JARRETT, J. T. & LANSBURY, P. T., JR. 1993. Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie? *Cell,* 73**,** 1055-8.

JEFFREY, M. & GONZALEZ, L. 2007. Classical sheep transmissible spongiform encephalopathies: pathogenesis, pathological phenotypes and clinical disease. *Neuropathol Appl Neurobiol,* 33**,** 373-94.

JEFFREY, M. & WELLS, G. A. 1988. Spongiform encephalopathy in a nyala (Tragelaphus angasi). *Vet Pathol,* 25**,** 398-9.

JEFFREY, M., BEGARA-MCGORUM, I., CLARK, S., MARTIN, S., CLARK, J., CHAPLIN, M. & GONZALEZ, L. 2002. 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,* 127**,** 264-73.

JEFFREY, M., GONZALEZ, L., CHONG, A., FOSTER, J., GOLDMANN, W., HUNTER, N. & MARTIN, S. 2006a. Ovine infection with the agents of scrapie (CH1641 isolate) and bovine spongiform encephalopathy: immunochemical similarities can be resolved by immunohistochemistry. *J Comp Pathol,* 134**,** 17-29.

JEFFREY, M., MARTIN, S. & GONZALEZ, L. 2003. Cell-associated variants of disease-specific prion protein immunolabelling are found in different sources of sheep transmissible spongiform encephalopathy. *J Gen Virol,* 84**,** 1033-45.

JEFFREY, M., MARTIN, S., GONZALEZ, L., FOSTER, J., LANGEVELD, J. P., VAN ZIJDERVELD, F. G., GRASSI, J. & HUNTER, N. 2006b. Immunohistochemical features of PrP(d) accumulation in natural and experimental goat transmissible spongiform encephalopathies. *J Comp Pathol,* 134**,** 171-81.

JEFFREY, M., MARTIN, S., GONZALEZ, L., RYDER, S. J., BELLWORTHY, S. J. & JACKMAN, R. 2001. Differential diagnosis of infections with the bovine spongiform encephalopathy (BSE) and scrapie agents in sheep. *J Comp Pathol,* 125**,** 271-84.

JONES, M., PEDEN, A. H., PROWSE, C. V., GRONER, A., MANSON, J. C., TURNER, M. L., IRONSIDE, J. W., MACGREGOR, I. R. & HEAD, M. W. 2007. In vitro amplification and detection of variant Creutzfeldt-Jakob disease PrPSc. *J Pathol,* 213**,** 21-6.

JONES, M., PEDEN, A. H., YULL, H., WIGHT, D., BISHOP, M. T., PROWSE, C. V., TURNER, M. L., IRONSIDE, J. W., MACGREGOR, I. R. & HEAD, M. W. 2009. Human platelets as a substrate source for the in vitro amplification

of the abnormal prion protein (PrP) associated with variant Creutzfeldt-Jakob disease. *Transfusion,* 49**,** 376-84.

KANAANI, J., PRUSINER, S.B., DIACOVO, J., BAEKKESKOV, S., LEGNAME, G. 2005. Recombinant prion protein induces rapid polarization and development of synapses in embryonic rat hippocampal neurons in vitro. *J Neurochem*, 95:1373–1386.

KIM, J. I., CALI, I., SUREWICZ, K., KONG, Q., RAYMOND, G. J., ATARASHI, R., RACE, B., QING, L., GAMBETTI, P., CAUGHEY, B. & SUREWICZ, W. K. 2010. Mammalian prions generated from bacterially expressed prion protein in the absence of any mammalian cofactors. *J Biol Chem,* 285**,** 14083-7.

KIM, J. I., SUREWICZ, K., GAMBETTI, P. & SUREWICZ, W. K. 2009. The role of glycophosphatidylinositol anchor in the amplification of the scrapie isoform of prion protein in vitro. *FEBS Lett,* 583**,** 3671-5.

KIMBERLIN, R. H., WALKER, C. A. 1978. Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. *J Gen Virol*., 39, 487–496.

KIRKWOOD, J. K. & CUNNINGHAM, A. A. 1994. Epidemiological observations on spongiform encephalopathies in captive wild animals in the British Isles. *Vet Rec,* 135**,** 296-303.

KIRKWOOD, J. K., CUNNINGHAM, A. A., WELLS, G. A., WILESMITH, J. W. & BARNETT, J. E. 1993. Spongiform encephalopathy in a herd of greater kudu (Tragelaphus strepsiceros): epidemiological observations. *Vet Rec,* 133**,** 360-4.

KIRKWOOD, J. K., WELLS, G. A., WILESMITH, J. W., CUNNINGHAM, A. A. & JACKSON, S. I. 1990. Spongiform encephalopathy in an arabian oryx (Oryx leucoryx) and a greater kudu (Tragelaphus strepsiceros). *Vet Rec,* 127**,** 418-20.

KLINGEBORN, M., RACE, B., MEADE-WHITE, K. D. & CHESEBRO, B. 2011. Lower specific infectivity of protease-resistant prion protein generated in cell-free reactions. *Proc Natl Acad Sci U S A,* 108**,** E1244-53.

KONOLD, T., BONE, G., RYDER, S., HAWKINS, S. A., COURTIN, F. & BERTHELIN-BAKER, C. 2004. Clinical findings in 78 suspected cases of bovine spongiform encephalopathy in Great Britain. *Vet Rec,* 155**,** 659-66.

KONOLD, T., LEE, Y. H., STACK, M. J., HORROCKS, C., GREEN, R. B. 2006. Different prion disease phenotypes result from inoculation of cattle with two temporally separated sources of sheep scrapie from Great Britain. *BMC Vet Res*., 2:31-51.

KONOLD, T., MOORE, S. J., BELLWORTHY, S. J. & SIMMONS, H. A. 2008. Evidence of scrapie transmission via milk. *BMC Vet Res,* 4**,** 14.

KRETZSCHMAR, H. A., TINGS, T., MADLUNG, A., GIESE, A. & HERMS, J. 2000. Function of PrP(C) as a copper-binding protein at the synapse. *Arch Virol Suppl***,** 239-49.

KRUGER, D., THOMZIG, A., LENZ, G., KAMPF, K., MCBRIDE, P. & BEEKES, M. 2009. Faecal shedding, alimentary clearance and intestinal spread of prions in hamsters fed with scrapie. *Vet Res,* 40**,** 4.

KUPFER, L., EIDEN, M., BUSCHMANN, A. & GROSCHUP, M. H. 2007. 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,* 1772**,** 704-13.

KURT, T. D., PERROTT, M. R., WILUSZ, C. J., WILUSZ, J., SUPATTAPONE, S., TELLING, G. C., ZABEL, M. D. & HOOVER, E. A. 2007. Efficient in vitro amplification of chronic wasting disease PrPRES. *J Virol,* 81**,** 9605-8.

LACROUX, C., SIMON, S., BENESTAD, S. L., MAILLET, S., MATHEY, J., LUGAN, S., CORBIERE, F., CASSARD, H., COSTES, P., BERGONIER, D., WEISBECKER, J. L., MOLDAL, T., SIMMONS, H., LANTIER, F., FERAUDET-TARISSE, C., MOREL, N., SCHELCHER, F., GRASSI, J. & ANDREOLETTI, O. 2008. Prions in milk from ewes incubating natural scrapie. *PLoS Pathog,* 4**,** e1000238.

LAEGREID, W. W., CLAWSON, M. L., HEATON, M. P., GREEN, B. T., O'ROURKE, K. I. & KNOWLES, D. P. 2008. Scrapie resistance in ARQ sheep. *J Virol,* 82**,** 10318-20.

LAMPO, E., VAN POUCKE, M., VANDESOMPELE, J., ERKENS, T., VAN ZEVEREN, A. & PEELMAN, L. J. 2009. Positive correlation between relative mRNA expression of PRNP and SPRN in cerebral and cerebellar cortex of sheep. *Mol Cell Probes,* 23**,** 60-4.

LEE, C. I., YANG, Q., PERRIER, V. & BASKAKOV, I. V. 2007. The dominant-negative effect of the Q218K variant of the prion protein does not require protein X. *Protein Sci,* 16**,** 2166-73.

LEZMI, S., MARTIN, S., SIMON, S., COMOY, E., BENCSIK, A., DESLYS, J. P., GRASSI, J., JEFFREY, M. & BARON, T. 2004. 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,* 78**,** 3654-62.

LIGIOS, C., CANCEDDA, M. G., MADAU, L., SANTUCCIU, C., MAESTRALE, C., AGRIMI, U., RU, G. & DI GUARDO, G. 2006. PrP(Sc) deposition in nervous tissues without lymphoid tissue involvement is frequently found in ARQ/ARQ Sarda breed sheep preclinically affected with natural scrapie. *Arch Virol,* 151**,** 2007-20.

LIGIOS, C., JEFFREY, M., RYDER, S. J., BELLWORTHY, S. J. & SIMMONS, M. M. 2002. Distinction of scrapie phenotypes in sheep by lesion profiling. *J Comp Pathol,* 127**,** 45-57.

LIGIOS, C., SIGURDSON, C. J., SANTUCCIU, C., CARCASSOLA, G., MANCO, G., BASAGNI, M., MAESTRALE, C., CANCEDDA, M. G., MADAU, L. & AGUZZI, A. 2005. PrPSc in mammary glands of sheep affected by scrapie and mastitis. *Nat Med,* 11**,** 1137-8.

LLOYD, S., MEAD, S. & COLLINGE, J. 2011. Genetics of prion disease. *Top Curr Chem,* 305**,** 1-22.

LUHKEN, G., BUSCHMANN, A., BRANDT, H., EIDEN, M., GROSCHUP, M. H. & ERHARDT, G. 2007. Epidemiological and genetical differences between classical and atypical scrapie cases. *Vet Res,* 38**,** 65-80.

MADDISON, B. C., BAKER, C. A., REES, H. C., TERRY, L. A., THORNE, L., BELLWORTHY, S. J., WHITELAM, G. C. & GOUGH, K. C. 2009. Prions are secreted in milk from clinically normal scrapie-exposed sheep. *J Virol,* 83**,** 8293-6.

MADDISON, B. C., BAKER, C. A., TERRY, L. A., BELLWORTHY, S. J., THORNE, L., REES, H. C. & GOUGH, K. C. 2010. Environmental sources of scrapie prions. *J Virol,* 84**,** 11560-2.

MADDISON, B. C., OWEN, J. P., BISHOP, K., SHAW, G., REES, H. C. & GOUGH, K. C. 2010. The interaction of ruminant PrP(Sc) with soils is influenced by prion source and soil type. *Environ Sci Technol,* 44**,** 8503-8.

MADDISON, B. C., OWEN, J. P., TAEMA, M. A., SHAW, G. & GOUGH, K. C. 2012. Temperature influences the interaction of ruminant PrP (TSE) with soil. *Prion,* 6.

MADDISON, B. C., REES, H. C., BAKER, C. A., TAEMA, M., BELLWORTHY, S. J., THORNE, L., TERRY, L. A. & GOUGH, K. C. 2010. Prions are secreted into the oral cavity in sheep with preclinical scrapie. *J Infect Dis,* 201**,** 1672-6.

MADDISON, B. C., WHITELAM, G. C. & GOUGH, K. C. 2007. Cellular prion protein in ovine milk. *Biochem Biophys Res Commun,* 353**,** 195-9.

MAKARAVA, N., OSTAPCHENKO, V. G., SAVTCHENKO, R. & BASKAKOV, I. V. 2009. Conformational switching within individual amyloid fibrils. *J Biol Chem,* 284**,** 14386-95.

MALUQUER DE MOTES, C., GRASSI, J., SIMON, S., HERVA, M. E., TORRES, J. M., PUMAROLA, M. & GIRONES, R. 2008. Excretion of BSE and scrapie prions in stools from murine models. *Vet Microbiol,* 131**,** 205-11.

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

MANUELIDIS, L., LIU, Y. & MULLINS, B. 2009. Strain-specific viral properties of variant Creutzfeldt-Jakob disease (vCJD) are encoded by the agent and not by host prion protein. *J Cell Biochem,* 106**,** 220-31.

MANUELIDIS, L., YU, Z. X., BARQUERO, N. & MULLINS, B. 2007. Cells infected with scrapie and Creutzfeldt-Jakob disease agents produce intracellular 25-nm virus-like particles. *Proc Natl Acad Sci U S A,* 104**,** 1965-70.

MARTIN, S., GONZALEZ, L., CHONG, A., HOUSTON, F. E., HUNTER, N. & JEFFREY, M. 2005. Immunohistochemical characteristics of disease-associated PrP are not altered by host genotype or route of inoculation following infection of sheep with bovine spongiform encephalopathy. *J Gen Virol,* 86**,** 839-48.

MASTRIANNI, J. A. 2004. Prion diseases. *Clinical Neuroscience Research,* 3**,** 469-480.

MASTRIANNI, J. A., NIXON, R., LAYZER, R., TELLING, G. C., HAN, D., DEARMOND, S. J. & PRUSINER, S. B. 1999. Prion protein conformation in a patient with sporadic fatal insomnia. *N Engl J Med,* 340**,** 1630-8.

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. 2009. Infectious prions in pre-clinical deer and transmission of chronic wasting disease solely by environmental exposure. *PLoS One,* 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. 2006. Infectious prions in the saliva and blood of deer with chronic wasting disease. *Science,* 314**,** 133-6.

MCCUTCHEON, S., HUNTER, N. & HOUSTON, F. 2005. Use of a new immunoassay to measure PrP Sc levels in scrapie-infected sheep brains reveals PrP genotype-specific differences. *J Immunol Methods,* 298**,** 119-28.

MCGUIRE, L.I., PEDEN, A.H., ORRÚ, C.D., WILHAM, J.M., APPLEFORD, N.E., MALLINSON, G., ANDREWS, M., HEAD, M.W., CAUGHEY, B., WILL R.G., KNIGHT, R.S., GREEN, A.J. (2012): Real time quaking-induced conversion analysis of cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease. Ann Neurol. 72(2):278-85

MCKINTOSH, E., TABRIZI, S. J. & COLLINGE, J. 2003. Prion diseases. *J Neurovirol,* 9**,** 183-93.

MONLEON, E., GARZA, M. C., SARASA, R., ALVAREZ-RODRIGUEZ, J., BOLEA, R., MONZON, M., VARGAS, M. A., BADIOLA, J. J. & ACIN, C. 2011. An assessment of the efficiency of PrPsc detection in rectal mucosa and third-eyelid biopsies from animals infected with scrapie. *Vet Microbiol,* 147**,** 237-43.

MOORE, R. A., TAUBNER, L. M. & PRIOLA, S. A. 2009. Prion protein misfolding and disease. *Curr Opin Struct Biol,* 19**,** 14-22.

MOORE, R. A., VORBERG, I. & PRIOLA, S. A. 2005. Species barriers in prion diseases--brief review. *Arch Virol Suppl***,** 187-202.

MOREL, E., FOUQUET, S., STRUP-PERROT, C., PICHOL THIEVEND, C., PETIT, C., LOEW, D., FAUSSAT, A. M., YVERNAULT, L., PINCON-RAYMOND, M., CHAMBAZ, J., ROUSSET, M., THENET, S. & CLAIR, C. 2008. The cellular prion protein PrP(c) is involved in the proliferation of epithelial cells and in the distribution of junction-associated proteins. *PLoS One,* 3**,** e3000.

MOREL, N., ANDREOLETTI, O., GRASSI, J. & CLEMENT, G. 2007. Absolute and relative quantification of sheep brain prion protein (PrP) allelic variants by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom,* 21**,** 4093-100.

MORENO, C. R., COSSEDDU, G. M., SCHIBLER, L., ROIG, A., MOAZAMI-GOUDARZI, K., ANDREOLETTI, O., EYCHENNE, F., LAJOUS, D., SCHELCHER, F., CRIBIU, E. P., LAURENT, P., VAIMAN, D. & ELSEN, J. M. 2008. Identification of new quantitative trait Loci (other than the *PRNP* gene) modulating the scrapie incubation period in sheep. *Genetics,* 179**,** 723-6.

MORENO, C. R., MOAZAMI-GOUDARZI, K., LAURENT, P., CAZEAU, G., ANDREOLETTI, O., CHADI, S., ELSEN, J. M. & CALAVAS, D. 2007. Which PrP haplotypes in a French sheep population are the most susceptible to atypical scrapie? *Arch Virol,* 152**,** 1229-32.

MURAYAMA, Y., YOSHIOKA, M., OKADA, H., TAKATA, M., YOKOYAMA, T. & MOHRI, S. 2007. Urinary excretion and blood level of prions in scrapie-infected hamsters. *J Gen Virol,* 88**,** 2890-8.

NAKAMITSU, S., KUROKAWA, A., YAMASAKI, T., URYU, M., HASEBE, R. & HORIUCHI, M. 2010. Cell density-dependent increase in the level of protease-resistant prion protein in prion-infected Neuro2a mouse neuroblastoma cells. *J Gen Virol,* 91**,** 563-9.

NICOT, S. & BARON, T. G. 2010. Strain-specific proteolytic processing of the prion protein in prion diseases of ruminants transmitted in ovine transgenic mice. *J Gen Virol,* 91**,** 570-4.

NONNO, R., ESPOSITO, E., VACCARI, G., CONTE, M., MARCON, S., DI BARI, M., LIGIOS, C., DI GUARDO, G. & AGRIMI, U. 2003. Molecular analysis of cases of Italian sheep scrapie and comparison with cases of bovine spongiform encephalopathy (BSE) and experimental BSE in sheep. *J Clin Microbiol,* 41**,** 4127-33.

NUSSBAUM, R. E., HENDERSON, W. M., PATTISON, I. H., ELCOCK, N. V. & DAVIES, D. C. 1975. The establishment of sheep flocks of predictable susceptibility to expirimental scrapie. *Res Vet Sci,* 18**,** 49-58.

OIE Terrestrial Manual 2009: "Scrapie, Chapter 2 . 7 . 1 3". Available on line: [http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.07.13_SCRAPIE.pdf]

O'ROURKE, K. I., BASZLER, T. V., BESSER, T. E., MILLER, J. M., CUTLIP, R. C., WELLS, G. A., RYDER, S. J., PARISH, S. M., HAMIR, A. N., COCKETT, N. E., JENNY, A. & KNOWLES, D. P. 2000. Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. *J Clin Microbiol,* 38**,** 3254-9.

O'ROURKE, K. I., BASZLER, T. V., PARISH, S. M. & KNOWLES, D. P. 1998. Preclinical detection of PrPSc in nictitating membrane lymphoid tissue of sheep. *Vet Rec,* 142**,** 489-91.

O'ROURKE, K. I., DUNCAN, J. V., LOGAN, J. R., ANDERSON, A. K., NORDEN, D. K., WILLIAMS, E. S., COMBS, B. A., STOBART, R. H., MOSS, G. E. & SUTTON, D. L. 2002. Active surveillance for scrapie by third eyelid biopsy and genetic susceptibility testing of flocks of sheep in Wyoming. *Clin Diagn Lab Immunol,* 9**,** 966-71.

ORRU, C. D., WILHAM, J. M., HUGHSON, A. G., RAYMOND, L. D., MCNALLY, K. L., BOSSERS, A., LIGIOS, C. & CAUGHEY, B. 2009. Human variant Creutzfeldt-Jakob disease and sheep scrapie PrPres detection using seeded conversion of recombinant prion protein. *Protein Eng Des Sel.*

ORRU, C. D., WILHAM, J. M., RAYMOND, L. D., KUHN, F., SCHROEDER, B., RAEBER, A. J. & CAUGHEY, B. 2011. Prion disease blood test using immunoprecipitation and improved quaking-induced conversion. *MBio,* 2**,** e00078-11.

ORRU, C. D., WILHAM, J. M., RAYMOND, L. D., KUHN, F., SCHROEDER, B., RAEBER, A. J. & CAUGHEY, B. 2011. Prion disease blood test using immunoprecipitation and improved quaking-induced conversion. *MBio,* 2**,** e00078-11.

OSTAPCHENKO, V. G., MAKARAVA, N., SAVTCHENKO, R. & BASKAKOV, I. V. 2008. The polybasic N-terminal region of the prion protein controls the

physical properties of both the cellular and fibrillar forms of PrP. *J Mol Biol,* 383**,** 1210-24.

OWEN, J. P., REES, H. C., MADDISON, B. C., TERRY, L. A., THORNE, L., JACKMAN, R., WHITELAM, G. C. & GOUGH, K. C. 2007. Molecular profiling of ovine prion diseases by using thermolysin-resistant PrPSc and endogenous C2 PrP fragments. *J Virol,* 81**,** 10532-9.

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. 1993. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci U S A,* 90**,** 10962-6.

PANZA, G., LUERS, L., STOHR, J., NAGEL-STEGER, L., WEISS, J., RIESNER, D., WILLBOLD, D. & BIRKMANN, E. 2010. Molecular interactions between prions as seeds and recombinant prion proteins as substrates resemble the biological interspecies barrier in vitro. *PLoS One,* 5**,** e14283.

PANZA, G., STOHR, J., DUMPITAK, C., PAPATHANASSIOU, D., WEISS, J., RIESNER, D., WILLBOLD, D. & BIRKMANN, E. 2008. Spontaneous and BSE-prion-seeded amyloid formation of full length recombinant bovine prion protein. *Biochem Biophys Res Commun,* 373**,** 493-7.

PARRY, H. B. 1962. Scrapie: a transmissible and hereditary disease of sheep. *Heredity,* 17**,** 75-105.

PASTRANA, M. A., SAJNANI, G., ONISKO, B., CASTILLA, J., MORALES, R., SOTO, C. & REQUENA, J. R. 2006. Isolation and characterization of a proteinase K-sensitive PrPSc fraction. *Biochemistry,* 45**,** 15710-7.

PATTISON, I. H. 1974. Scrapie in sheep selectively bred for high susceptibility. *Nature,* 248**,** 594-5.

PATTISON, I. H., HOARE, M. N., JEBBETT, J. N. & WATSON, W. A. 1972. Spread of scrapie to sheep and goats by oral dosing with foetal membranes from scrapie-affected sheep. *Vet Rec,* 90**,** 465-8.

PEDEN, A. H., MCGUIRE, L. I., APPLEFORD, N. E., MALLINSON, G., WILHAM, J. M., ORRU, C. D., CAUGHEY, B., IRONSIDE, J. W., KNIGHT, R. S., WILL, R. G., GREEN, A. J. & HEAD, M. W. 2012. Sensitive and specific detection of sporadic Creutzfeldt-Jakob disease brain

prion protein using real-time quaking-induced conversion. *J Gen Virol,* 93**,** 438-49.

PIRISINU, L., MIGLIORE, S., DI BARI, M. A., ESPOSITO, E., BARON, T., D'AGOSTINO, C., DE GROSSI, L., VACCARI, G., AGRIMI, U. & NONNO, R. 2011. Molecular discrimination of sheep bovine spongiform encephalopathy from scrapie. *Emerg Infect Dis,* 17**,** 695-8.

PIRO, J. R., WANG, F., WALSH, D. J., REES, J. R., MA, J. & SUPATTAPONE, S. 2011. Seeding specificity and ultrastructural characteristics of infectious recombinant prions. *Biochemistry,* 50**,** 7111-6.

PRIEM, J., LANGEVELD, J., VAN ZIJDERVELD, F., BOSSERS, A., (2007) Ovine and bovine species/polymorphism-specificity of the protein misfolding cyclic amplification (PMCA). Wageningen University and Researchcenter Publications, Netherlands.

PRINZ, M., HEIKENWALDER, M., SCHWARZ, P., TAKEDA, K., AKIRA, S. & AGUZZI, A. 2003. Prion pathogenesis in the absence of Toll-like receptor signalling. *EMBO Rep,* 4**,** 195-9.

PRUSINER, S. B. 1982. Research on scrapie. *Lancet,* 2**,** 494-5.

PRUSINER, S. B. 1991. Molecular biology of prion diseases. *Science,* 252**,** 1515-22.

PRUSINER, S. B. 1997. Prion diseases and the BSE crisis. *Science,* 278**,** 245-51.

PRUSINER, S. B. 1998. Prions. *Proc Natl Acad Sci U S A,* 95**,** 13363-83.

REZAEI, H., MARC, D., CHOISET, Y., TAKAHASHI, M., HUI BON HOA, G., HAERTLE, T., GROSCLAUDE, J. & DEBEY, P. 2000. High yield purification and physico-chemical properties of full-length recombinant allelic variants of sheep prion protein linked to scrapie susceptibility. *Eur J Biochem,* 267**,** 2833-9.

RICHT, J. A. & HALL, S. M. 2008. BSE case associated with prion protein gene mutation. *PLoS Pathog,* 4**,** e1000156.

RIESNER, D. 2003. Biochemistry and structure of PrP(C) and PrP(Sc). *Br Med Bull,* 66**,** 21-33.

ROUCOU, X., GAINS, M., LEBLANC, A.C. 2004. Neuroprotective functions of prion protein. *J Neurosci Res,* 75:153–161.

ROUCOU, X., LEBLANC, A.C. 2005. Cellular prion protein neuroprotective function: implications in prion diseases. *J Mol Med,* 83:3–11.

RUBENSTEIN, R., CHANG, B., GRAY, P., PILTCH, M., BULGIN, M. S., SORENSEN-MELSON, S. & MILLER, M. W. 2010. A novel method for preclinical detection of PrPSc in blood. *J Gen Virol,* 91**,** 1883-92.

RUBENSTEIN, R., SCALICI, C. L., PAPINI, M. C., CALLAHAN, S. M. & CARP, R. I. 1990. Further characterization of scrapie replication in PC12 cells. *J Gen Virol,* 71 (Pt 4)**,** 825-31.

RYOU, C. & MAYS, C. E. 2008. Prion propagation in vitro: are we there yet? *Int J Med Sci,* 5**,** 347-53.

SAA, P., CASTILLA, J. & SOTO, C. 2006. Presymptomatic detection of prions in blood. *Science,* 313**,** 92-4.

SABORIO, G. P., PERMANNE, B. & SOTO, C. 2001. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature,* 411**,** 810-3.

SAFAR, J. & PRUSINER, S. B. 1998. Molecular studies of prion diseases. *Prog Brain Res,* 117**,** 421-34.

SAFAR, J. G., KELLINGS, K., SERBAN, A., GROTH, D., CLEAVER, J. E., PRUSINER, S. B. & RIESNER, D. 2005. Search for a prion-specific nucleic acid. *J Virol,* 79**,** 10796-806.

SAFAR, J. G., LESSARD, P., TAMGUNEY, G., FREYMAN, Y., DEERING, C., LETESSIER, F., DEARMOND, S. J. & PRUSINER, S. B. 2008. Transmission and detection of prions in feces. *J Infect Dis,* 198**,** 81-9.

SAFAR, J. G., SCOTT, M., MONAGHAN, J., DEERING, C., DIDORENKO, S., VERGARA, J., BALL, H., LEGNAME, G., LECLERC, E., SOLFOROSI, L., SERBAN, H., GROTH, D., BURTON, D. R., PRUSINER, S. B. & WILLIAMSON, R. A. 2002. Measuring prions causing bovine spongiform encephalopathy or chronic wasting disease by immunoassays and transgenic mice. *Nat Biotechnol,* 20**,** 1147-50.

SAFAR, J., WILLE, H., ITRI, V., GROTH, D., SERBAN, H., TORCHIA, M., COHEN, F. E. & PRUSINER, S. B. 1998. Eight prion strains have PrP(Sc) molecules with different conformations. *Nat Med,* 4**,** 1157-65.

SANTUCCIONE, A., SYTNYK, V., LESHCHYNS'KA, I., SCHACHNER, M., 2005. Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J Cell Biol*, 169:341–354

SAUNDERS, G. C., CAWTHRAW, S., MOUNTJOY, S. J., HOPE, J. & WINDL, O. 2006. PrP genotypes of atypical scrapie cases in Great Britain. *J Gen Virol,* 87**,** 3141-9.

SAUNDERS, S. E., BARTELT-HUNT, S. L. & BARTZ, J. C. 2012. Occurrence, transmission, and zoonotic potential of chronic wasting disease. *Emerg Infect Dis,* 18**,** 369-76.

SEEGER, H., HEIKENWALDER, M., ZELLER, N., KRANICH, J., SCHWARZ, P., GASPERT, A., SEIFERT, B., MIELE, G. & AGUZZI, A. 2005. Coincident scrapie infection and nephritis lead to urinary prion excretion. *Science,* 310**,** 324-6.

SIGURDSON, C. J. & MILLER, M. W. 2003. Other animal prion diseases. *Br Med Bull,* 66**,** 199-212.

SINGH, A., MOHAN, M. L., ISAAC, A. O., LUO, X., PETRAK, J., VYORAL, D. & SINGH, N. 2009. Prion protein modulates cellular iron uptake: a novel function with implications for prion disease pathogenesis. *PLoS One,* 4**,** e4468.

SMIRNOVAS, V., KIM, J. I., LU, X., ATARASHI, R., CAUGHEY, B. & SUREWICZ, W. K. 2009. Distinct structures of scrapie prion protein (PrPSc)-seeded versus spontaneous recombinant prion protein fibrils revealed by hydrogen/deuterium exchange. *J Biol Chem,* 284**,** 24233-41.

SOTO, C., ANDERES, L., SUARDI, S., CARDONE, F., CASTILLA, J., FROSSARD, M. J., PEANO, S., SAA, P., LIMIDO, L., CARBONATTO, M., IRONSIDE, J., TORRES, J. M., POCCHIARI, M. & TAGLIAVINI, F. 2005. Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. *FEBS Lett,* 579**,** 638-42.

SOTO, C., SABORIO, G. P. & ANDERES, L. 2002. Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. *Trends Neurosci,* 25**,** 390-4.

SPINNER, D. S., CHO, I. S., PARK, S. Y., KIM, J. I., MEEKER, H. C., YE, X., LAFAUCI, G., KERR, D. J., FLORY, M. J., KIM, B. S., KASCSAK, R. B., WISNIEWSKI, T., LEVIS, W. R., SCHULLER-LEVIS, G. B., CARP, R. I., PARK, E. & KASCSAK, R. J. 2008. Accelerated prion disease pathogenesis in Toll-like receptor 4 signaling-mutant mice. *J Virol,* 82**,** 10701-8.

SPINNER, D. S., KASCSAK, R. B., LAFAUCI, G., MEEKER, H. C., YE, X., FLORY, M. J., KIM, J. I., SCHULLER-LEVIS, G. B., LEVIS, W. R., WISNIEWSKI, T., CARP, R. I. & KASCSAK, R. J. 2007. CpG oligodeoxynucleotide-enhanced humoral immune response and production of antibodies to prion protein PrPSc in mice immunized with 139A scrapie-associated fibrils. *J Leukoc Biol,* 81**,** 1374-85.

SPIROPOULOS, J., CASALONE, C., CARAMELLI, M. & SIMMONS, M. M. 2007. Immunohistochemistry for PrPSc in natural scrapie reveals patterns which are associated with the PrP genotype. *Neuropathol Appl Neurobiol,* 33**,** 398-409.

SPIROPOULOS, J., LOCKEY, R., SALLIS, R. E., TERRY, L. A., THORNE, L., HOLDER, T. M., BECK, K. E. & SIMMONS, M. M. 2011. Isolation of prion with BSE properties from farmed goat. *Emerg Infect Dis,* 17**,** 2253-61.

STACK, M. J., CHAPLIN, M. J. & CLARK, J. 2002. 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,* 104**,** 279-86.

STACK, M., FOCOSI-SNYMAN, R., CAWTHRAW, S., DAVIS, L., JENKINS, R., THORNE, L., CHAPLIN, M., EVERITT, S., SAUNDERS, G. & TERRY, L. 2009. Two unusual bovine spongiform encephalopathy cases detected in Great Britain. *Zoonoses Public Health,* 56**,** 376-83.

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. 2006. Monitoring for bovine spongiform encephalopathy in sheep in Great Britain, 1998-2004. *J Gen Virol,* 87**,** 2099-107.

STEWART, P., SHEN, C., ZHAO, D. & GOLDMANN, W. 2009. Genetic analysis of the SPRN gene in ruminants reveals polymorphisms in the alanine-rich segment of shadoo protein. *J Gen Virol,* 90**,** 2575-80.

SUREWICZ, W. K., JONES, E. M. & APETRI, A. C. 2006. The emerging principles of mammalian prion propagation and transmissibility barriers: Insight from studies in vitro. *Acc Chem Res,* 39**,** 654-62.

SWEENEY, T., KUCZIUS, T., MCELROY, M., PARADA, M. G. & GROSCHUP, M. H. 2000. Molecular analysis of Irish sheep scrapie cases. *J Gen Virol,* 81**,** 2121.

SWEETING, B., KHAN, M. Q., CHAKRABARTTY, A., PAI, E. F. 2010. Structural factors underlying the species barrier and susceptibility to infection in prion disease. *Biochem Cell Biol.* 88, 2:195-202.

TAMGUNEY, G., GILES, K., GLIDDEN, D. V., LESSARD, P., WILLE, H., TREMBLAY, P., GROTH, D. F., YEHIELY, F., KORTH, C., MOORE, R. C., TATZELT, J., RUBINSTEIN, E., BOUCHEIX, C., YANG, X., STANLEY, P., LISANTI, M. P., DWEK, R. A., RUDD, P. M., MOSKOVITZ, J., EPSTEIN, C. J., CRUZ, T. D., KUZIEL, W. A., MAEDA, N., SAP, J., ASHE, K. H., CARLSON, G. A., TESSEUR, I., WYSS-CORAY, T., MUCKE, L., WEISGRABER, K. H., MAHLEY, R. W., COHEN, F. E. & PRUSINER, S. B. 2008. Genes contributing to prion pathogenesis. *J Gen Virol,* 89**,** 1777-88.

TELLING, G. C. 2004. The mechanism of prion strain propagation. *Genome Biol,* 5**,** 222.

TERRY, L. A., HOWELLS, L., BISHOP, K., BAKER, C. A., EVEREST, S., THORNE, L., MADDISON, B. C. & GOUGH, K. C. 2011. Detection of prions in the faeces of sheep naturally infected with classical scrapie. *Vet Res,* 42**,** 65.

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. 2009. Detection of PrPsc in blood from sheep infected with the scrapie and bovine spongiform encephalopathy agents. *J Virol,* 83**,** 12552-8.

TERRY, L. A., MARSH, S., RYDER, S. J., HAWKINS, S. A., WELLS, G. A. & SPENCER, Y. I. 2003. Detection of disease-specific PrP in the distal ileum of cattle exposed orally to the agent of bovine spongiform encephalopathy. *Vet Rec,* 152**,** 387-92.

THACKRAY, A. M., HOPKINS, L. & BUJDOSO, R. 2007. Proteinase K-sensitive disease-associated ovine prion protein revealed by conformation-dependent immunoassay. *Biochem J,* 401**,** 475-83.

THOMZIG, A., SCHULZ-SCHAEFFER, W., WREDE, A., WEMHEUER, W., BRENIG, B., KRATZEL, C., LEMMER, K. & BEEKES, M. 2007. Accumulation of pathological prion protein PrPSc in the skin of animals with experimental and natural scrapie. *PLoS Pathog,* 3**,** e66.

THORGEIRSDOTTIR, S., SIGURDARSON, S., THORISSON, H. M., GEORGSSON, G. & PALSDOTTIR, A. 1999. PrP gene polymorphism and natural scrapie in Icelandic sheep. *J Gen Virol,* 80 (Pt 9)**,** 2527-34.

THORNE, L. & TERRY, L. A. 2008. In vitro amplification of PrPSc derived from the brain and blood of sheep infected with scrapie. *J Gen Virol,* 89**,** 3177-84.

THURING, C. M., ERKENS, J. H., JACOBS, J. G., BOSSERS, A., VAN KEULEN, L. J., GARSSEN, G. J., VAN ZIJDERVELD, F. G., RYDER, S. J., GROSCHUP, M. H., SWEENEY, T. & LANGEVELD, J. P. 2004. Discrimination between scrapie and bovine spongiform encephalopathy in sheep by molecular size, immunoreactivity, and glycoprofile of prion protein. *J Clin Microbiol,* 42**,** 972-80.

TRANULIS, M. A., BENESTAD, S. L., BARON, T. & KRETZSCHMAR, H. 2011. Atypical prion diseases in humans and animals. *Top Curr Chem,* 305**,** 23-50.

TSE EU Community Reference Laboratory, 2007: Fresh Tissue Diagnosis of Transmissible Spongiform Encephalopathies (TSE's) in Cattle and Sheep. [vla.defra.gov.uk/science/docs/sci_Western.pdf].

TUO, W., O'ROURKE, K. I., ZHUANG, D., CHEEVERS, W. P., SPRAKER, T. R. & KNOWLES, D. P. 2002. Pregnancy status and fetal prion genetics determine PrPSc accumulation in placentomes of scrapie-infected sheep. *Proc Natl Acad Sci U S A,* 99**,** 6310-5.

TUO, W., ZHUANG, D., KNOWLES, D. P., CHEEVERS, W. P., SY, M. S. & O'ROURKE, K. I. 2001. Prp-c and Prp-Sc at the fetal-maternal interface. *J Biol Chem,* 276**,** 18229-34.

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. 2008. Host PrP glycosylation: a major factor determining the outcome of prion infection. *PLoS Biol,* 6**,** e100.

TZABAN, S., FRIEDLANDER, G., SCHONBERGER, O., HORONCHIK, L., YEDIDIA, Y., SHAKED, G., GABIZON, R. & TARABOULOS, A. 2002.

Protease-sensitive scrapie prion protein in aggregates of heterogeneous sizes. *Biochemistry,* 41**,** 12868-75.

USHIKI-KAKU, Y., ENDO, R., IWAMARU, Y., SHIMIZU, Y., IMAMURA, M., MASUJIN, K., YAMAMOTO, T., HATTORI, S., ITOHARA, S., IRIE, S. & YOKOYAMA, T. 2010. Tracing conformational transition of abnormal prion proteins during interspecies transmission by using novel antibodies. *J Biol Chem,* 285**,** 11931-6.

VAN KEULEN, L. J., BOSSERS, A. & VAN ZIJDERVELD, F. 2008. TSE pathogenesis in cattle and sheep. *Vet Res,* 39**,** 24.

VAN KEULEN, L. J., SCHREUDER, B. E., MELOEN, R. H., MOOIJ-HARKES, G., VROMANS, M. E. & LANGEVELD, J. P. 1996. Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. *J Clin Microbiol,* 34**,** 1228-31.

VAN KEULEN, L. J., SCHREUDER, B. E., VROMANS, M. E., LANGEVELD, J. P. & SMITS, M. A. 1999. Scrapie-associated prion protein in the gastrointestinal tract of sheep with natural scrapie. *J Comp Pathol,* 121**,** 55-63.

VILES, J. H., COHEN, F. E., PRUSINER, S. B., GOODIN, D. B., WRIGHT, P. E. & DYSON, H. J. 1999. Copper binding to the prion protein: structural implications of four identical cooperative binding sites. *Proc Natl Acad Sci U S A,* 96**,** 2042-7.

VORBERG, I., RAINES, A. & PRIOLA, S. A. 2004. Acute formation of protease-resistant prion protein does not always lead to persistent scrapie infection in vitro. *J Biol Chem,* 279**,** 29218-25.

VULIN, J., BIACABE, A. G., CAZEAU, G., CALAVAS, D. & BARON, T. 2011. Molecular typing of protease-resistant prion protein in transmissible spongiform encephalopathies of small ruminants, France, 2002-2009. *Emerg Infect Dis,* 17**,** 55-63.

WADSWORTH, J. D., JOINER, S., HILL, A. F., CAMPBELL, T. A., DESBRUSLAIS, M., LUTHERT, P. J. & COLLINGE, J. 2001. Tissue distribution of protease resistant prion protein in variant Creutzfeldt-Jakob disease using a highly sensitive immunoblotting assay. *Lancet,* 358**,** 171-80.

WADSWORTH, J. D., JOINER, S., LINEHAN, J. M., COOPER, S., POWELL, C., MALLINSON, G., BUCKELL, J., GOWLAND, I., ASANTE, E. A., BUDKA, H., BRANDNER, S. & COLLINGE, J. 2006. Phenotypic

heterogeneity in inherited prion disease (P102L) is associated with differential propagation of protease-resistant wild-type and mutant prion protein. *Brain,* 129**,** 1557-69.

WANG, F., WANG, X. & MA, J. 2011. Conversion of bacterially expressed recombinant prion protein. *Methods,* 53**,** 208-13.

WANG, F., WANG, X., YUAN, C. G. & MA, J. 2010. Generating a prion with bacterially expressed recombinant prion protein. *Science,* 327**,** 1132-5.

WANG, Y., QIN, Z., QIAO, J. & ZHAO, D. 2008. Polymorphisms of the prion protein gene in sheep of Inner Mongolia, China. *Virus Genes,* 37**,** 128-30.

WATANABE, T., YASUTAKA, Y., NISHIOKU, T., KUSAKABE, S., FUTAGAMI, K., YAMAUCHI, A. & KATAOKA, Y. 2011. Involvement of the cellular prion protein in the migration of brain microvascular endothelial cells. *Neurosci Lett*.

WATTS, J. C., DRISALDI, B., NG, V., YANG, J., STROME, B., HORNE, P., SY, M. S., YOONG, L., YOUNG, R., MASTRANGELO, P., BERGERON, C., FRASER, P. E., CARLSON, G. A., MOUNT, H. T., SCHMITT-ULMS, G. & WESTAWAY, D. 2007. The CNS glycoprotein Shadoo has PrP(C)-like protective properties and displays reduced levels in prion infections. *EMBO J,* 26**,** 4038-50.

WEBB, P. R., POWELL, L., DENYER, M., MARSH, S., WEAVER, C., SIMMONS, M. M., JOHNS, E., SHEEHAN, J., HORSFIELD, P., LYTH, C., WILSON, C., LONG, A., CAWTHRAW, S., SAUNDERS, G. C. & SPENCER, Y. I. 2009. A retrospective immunohistochemical study reveals atypical scrapie has existed in the United Kingdom since at least 1987. *J Vet Diagn Invest,* 21**,** 826-9.

WELLS, G. A., SCOTT, A. C., JOHNSON, C. T., GUNNING, R. F., HANCOCK, R. D., JEFFREY, M., DAWSON, M. & BRADLEY, R. 1987. A novel progressive spongiform encephalopathy in cattle. *Vet Rec,* 121**,** 419-20.

WELLS, G. A., SPIROPOULOS, J., HAWKINS, S. A. & RYDER, S. J. 2005. Pathogenesis of experimental bovine spongiform encephalopathy: preclinical infectivity in tonsil and observations on the distribution of lingual tonsil in slaughtered cattle. *Vet Rec,* 156**,** 401-7.

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

WILESMITH, J. W., RYAN, J. B. & ATKINSON, M. J. 1991. Bovine spongiform encephalopathy: epidemiological studies on the origin. *Vet Rec,* 128**,** 199-203.

WILHAM, J. M., ORRU, C. D., BESSEN, R. A., ATARASHI, R., SANO, K., RACE, B., MEADE-WHITE, K. D., TAUBNER, L. M., TIMMES, A. & CAUGHEY, B. 2010. Rapid end-point quantitation of prion seeding activity with sensitivity comparable to bioassays. *PLoS Pathog,* 6**,** e1001217.

WILL, R. G., IRONSIDE, J. W., ZEIDLER, M., COUSENS, S. N., ESTIBEIRO, K., ALPEROVITCH, A., POSER, S., POCCHIARI, M., HOFMAN, A. & SMITH, P. G. 1996. A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet,* 347**,** 921-5.

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

WILLIAMS, E. S. & YOUNG, S. 1992. Spongiform encephalopathies in Cervidae. *Rev Sci Tech,* 11**,** 551-67.

WILLOUGHBY, K., KELLY, D. F., LYON, D. G. & WELLS, G. A. 1992. Spongiform encephalopathy in a captive puma (Felis concolor). *Vet Rec,* 131**,** 431-4.

WOOD, J. L., MCGILL, I. S., DONE, S. H. & BRADLEY, R. 1997. Neuropathology of scrapie: a study of the distribution patterns of brain lesions in 222 cases of natural scrapie in sheep, 1982-1991. *Vet Rec,* 140**,** 167-74.

YOKOYAMA, T., MASUJIN, K., SCHMERR, M. J., SHU, Y., OKADA, H., IWAMARU, Y., IMAMURA, M., MATSUURA, Y., MURAYAMA, Y. & MOHRI, S. 2010. Intraspecies prion transmission results in selection of sheep scrapie strains. *PLoS One,* 5**,** e15450.

ZIVNY, J. H., GELDERMAN, M. P., XU, F., PIPER, J., HOLADA, K., SIMAK, J. & VOSTAL, J. G. 2008. Reduced erythroid cell and erythropoietin production in response to acute anemia in prion protein-deficient (Prnp-/-) mice. *Blood Cells Mol Dis,* 40**,** 302-7.