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Chronic wasting disease in Europe: characterisation of genetic susceptibility and strain diversity.

Amy Louise Robinson

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Doctor of Philosophy (PhD)
The University of Edinburgh

Declaration:

I declare that the work presented in this thesis is my own; all experiments were designed in collaboration with my supervisors and performed and analysed by myself unless otherwise clearly stated in the text. Part of this work has been previously published but none of the work presented in this thesis has been submitted for any other degree or professional qualification.

Amy Robinson

Abstract

Chronic wasting disease (CWD) is a prion disease that causes a fatal, progressive neurodegenerative disorder in cervid species including deer, elk, reindeer and moose. CWD has become widespread in North America in both wild and farmed cervid populations; disease control poses significant challenges due to efficient horizontal transmission and environmental persistence. The recent identification of CWD affecting reindeer, moose and red deer in Norway and other Scandinavian countries threatens a similar pattern of spread in European cervids, impacting the health of wild populations as well as deer farming and hunting industries. It is widely accepted that prion diseases are associated with an unusual infectious agent, a misfolded, protease resistant, self-propagating isoform (PrP^{Sc}) of the normal cellular prion protein (PrP^C). Susceptibility is largely determined by the sequence of the prion protein gene (*PRNP*). In North America, *PRNP* polymorphisms in cervid species have been associated with reduced susceptibility, altered pathogenesis and the emergence of new CWD strains.

Understanding CWD in the European context cannot be solely based on North American evidence due to the differences in cervid species and unknown effects of novel strains. I aimed to characterise the *PRNP* polymorphisms in British deer species and predict their effects on CWD susceptibility using *in-vitro* assays. I also aimed to determine if the pathological phenotypic characteristics of Norwegian and Canadian CWD isolates are maintained in primary passage to wild-type mice, and if lymphoid replication is an important feature in primary passage.

To determine the *PRNP* variation in British cervid species, a large-scale survey of the six most numerous species of wild deer in the UK was performed, including red (*Cervus elaphus*), sika (*Cervus nippon*), roe (*Capreolus capreolus*), fallow (*Dama dama*), muntjac (*Muntiacus reevesii*), and Chinese water deer (*Hydropotes inermis*), which was compared with two smaller surveys of red deer from Norway and the Czech Republic. The *PRNP* gene is well conserved in cervid

species, and we identified low levels of variation in most cervid species, particularly roe deer, which were identical in all individuals analysed. Based on comparison with susceptible North American deer species, it is likely that a high proportion of the UK deer population would be susceptible at least to the strains of CWD present in North America. Red deer showed the most *PRNP* variation, with five haplotypes identified which varied in frequency depending on location: the P168S and I247L polymorphisms were only identified in Scottish and Czech populations, respectively. These novel polymorphisms are not present in North American cervid populations, and their impact on CWD susceptibility are unknown.

The effects of *PRNP* variants on CWD susceptibility may be determined in naturally infected cervid populations, experimental challenge of cervids, or in transgenic mice expressing cervid prion proteins. The mechanisms by which individual amino acid substitutions influence disease susceptibility and pathogenesis are likely to include modification of epitopes crucial for PrP^C structure, stability, and conversion to PrP^{Sc}. These effects may be reproduced using *in-vitro* assays that analyse the conversion efficiency of different PrP protein sequences by PrP^{Sc} derived from prion-infected tissues. To test the misfolding capacity and conversion efficiency of British deer *PRNP* variants, two *in-vitro* assays were developed: a recombinant PrP protein fibrillisation assay and a cell-lysate protein misfolding amplification assay (PMCA) using RK13 cells transfected with red deer *PRNP* sequences as substrate.

Analysis of Norwegian CWD isolates and their transmission into rodent models has provided preliminary evidence that there are several different strains present in reindeer and moose which differ from those in North America. The gold standard test for strain differentiation is bioassay and assessment of the differences in heritable phenotypes in a new host species. As part of ongoing strain-typing studies of Norwegian CWD, I describe the primary passage of isolates from the first Norwegian reindeer and moose cases, as well as two Canadian CWD isolates, in well-defined panels of wild-type mice (VM, C57BL/6J and RIII) which have been used extensively in previous strain-typing

experiments. Evidence of transmission was examined in both brain and lymphoid tissues using a combination of immunohistochemistry, western blot and RT-QuIC amplification. Low transmission rates were observed for the transmission of natural CWD cases from Norway and Canada, however transmission rates were high for an experimental H95+ strain isolate with 72% of mice showing evidence of infection, 40% of which exhibited clinical signs.

The work presented here represents some of the first steps towards understanding *PRNP* variation in European cervid populations, developing methods for the assessment of novel *PRNP* polymorphisms, and the characterisation of European strain diversity. It is unclear if the efforts made to contain CWD in Norway will be successful, and further research is urgently required to inform risk assessments and control strategies. The origins of CWD remain unknown, and it is possible that further novel instances of CWD will be identified in different cervid species and geographic locations. The emergence of CWD in Europe poses a considerable challenge, the extent of which will critically depend on the susceptibility of cervid populations and the characterisation of new prion strains that may differ in their ability to transmit to other species including livestock and man.

Lay summary

Chronic wasting disease (CWD) is an infectious degenerative brain disease, similar to bovine spongiform encephalopathy (BSE) in cows and scrapie in sheep, that affects deer and related species including elk, reindeer and moose. Since its recognition in the late 1960s, it has become widespread across North America due to efficient transmission from deer to deer, persistence of infection in the environment, and the presence of disease in both wild and farmed deer populations. In 2016 CWD was recognised for the first time in a free-ranging Norwegian reindeer, and since then more cases have been found in reindeer, red deer and moose in Scandinavian countries. The likely spread of CWD throughout Europe is cause for concern due to the impact on wild deer health

and the deer farming and hunting industries. Due to the differences in deer species, and the characteristics of this new type of CWD identified in Europe, the lessons learned from the experience of CWD in North America do not necessarily apply to CWD in Europe. CWD and related diseases are caused by unusual infectious agents, consisting of misfolded proteins called prions. The building blocks (amino acids) of the prion protein (PrP) determine how easily it misfolds, and their makeup can vary according to the DNA sequence of the prion protein gene (*PRNP*), which is therefore an important factor in determining which animals will be vulnerable to infection. In North America, variation in the *PRNP* sequence has been linked to reduced susceptibility to CWD as well as differences in disease characteristics.

To determine the *PRNP* variation in British deer species, a large-scale survey of the six most numerous species of wild deer in the UK was performed, including red, sika, roe, fallow, muntjac, and Chinese water deer, and compared with two smaller surveys of red deer from Norway and the Czech Republic. We observed low levels of *PRNP* variation in the species analysed, particularly in roe deer, which were all identical. Comparing the *PRNP* sequences between British deer and susceptible North American species, it is likely that the majority would be susceptible to CWD originating in North America. Red deer had the most *PRNP* variation, with five different *PRNP* variants identified that varied depending on location. Two of these variants, identified in either Scottish or Czech red deer populations, are not present in North American deer species, and their effect on susceptibility is unknown. The effects of *PRNP* variants on susceptibility are usually determined by studying natural cases of CWD, or by experimental transmission into deer or mice expressing known variants of the prion protein. Different *PRNP* variants may determine how easily the protein misfolds into the disease-associated form, and this conversion process can be mimicked by simpler laboratory tests. To determine the effects of the British *PRNP* variants on susceptibility to CWD, we developed two such tests to study the conversion of the new PrP variants in European red deer.

Analysis of the CWD cases in Norway, and transmission of infected brain material into rodent models, suggests that the characteristics of disease in Norwegian cases are very different from North American cases, indicating that different 'strains' of CWD may be involved. As part of ongoing work to characterise the strains of CWD in Norway, brain material from Norwegian reindeer and moose, as well as two Canadian CWD cases, were inoculated in mice. Mice have been used extensively in previous experiments to characterise different prions from a variety of human and animal prion diseases. Low rates of transmission were observed from the transmission of natural cases of CWD from Norway and Canada, however, transmission of an experimental case of a different strain had much higher levels of transmission, with 72% of mice showing evidence of prion disease.

The work in this thesis represents some of the first steps towards understanding the *PRNP* variation in European deer populations, methods for the prediction of the effects of new deer prion protein variants on CWD susceptibility, and the analysis of CWD strains in Norway and Canada. It is unclear if the efforts made to contain CWD in Norway will be successful, and further research is urgently required to inform risk assessments and control strategies. The origins of CWD remain unknown, and it is possible that further new cases of CWD will be identified in different cervid species and geographic locations. The emergence of CWD in Europe poses a considerable challenge, the extent of which will critically depend on the susceptibility of deer populations and the assessment of new prion strains that may differ in their ability to infect other species such as cattle, sheep and humans.

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Contents

Declaration:	i
Abstract	ii
Lay summary	iv
Acknowledgements	vii
Contents	viii
Figure contents	xiv
Table contents	xvii
Abbreviations	xix
1 Introduction	1
1.1 An introduction to transmissible spongiform encephalopathies	2
1.1.1 General introduction	2
1.1.2 The history of prion diseases.....	2
1.1.3 New and emerging threats	3
1.1.4 Atypical prion diseases	4
1.2 The nature of the TSE agent	6
1.2.1 The prion hypothesis.....	6
1.2.2 Isoforms of the prion protein	7
1.2.3 Prions and proteinaceous nucleating particles.....	8
1.3 The prion protein in health and disease	8
1.3.1 Structure and cleavage of PrP ^C	9
1.3.2 Function of PrP ^C	10
1.3.3 Conversion of PrP ^C to PrP ^{Sc}	10
1.3.4 Detection of PrP ^{Sc}	11
1.3.5 In-vitro amplification of PrP ^{Sc}	12
1.3.5.1 Protein misfolding cyclic amplification (PMCA).....	13
1.3.5.2 Real-time Quaking Induced Conversion (RT-QuIC).....	13
1.3.6 Pathogenesis of prion disease.....	15
1.4 The prion protein gene and TSE susceptibility.....	16
1.4.1 The prion protein gene	16
1.4.2 PRNP genetics and prion disease	17
1.4.3 PRNP genetics and species barriers	19
1.5 Prion strain characteristics.....	20

1.6	Chronic wasting disease.....	22
1.6.1	<i>Origins and introduction</i>	22
1.6.2	<i>Distribution, transmission, and control</i>	23
1.6.3	<i>Diagnosis of CWD</i>	25
1.6.3.1	<i>Clinical signs</i>	25
1.6.3.2	<i>Differential diagnoses, gross and clinical pathology</i>	26
1.6.3.3	<i>Diagnostic tests</i>	26
1.6.4	<i>Experimental transmissions and species barriers</i>	28
1.6.4.1	<i>Cervids</i>	28
1.6.4.2	<i>Other animal hosts</i>	29
1.6.4.3	<i>Humans</i>	29
1.6.5	<i>Cervid PrP genetics</i>	31
1.6.5.1	<i>Effects of cervid prion protein gene (PRNP) variation on susceptibility</i>	31
1.6.5.2	<i>Cervid PRNP variation and protein structure</i>	34
1.6.5.3	<i>PRNP polymorphisms and disease management</i>	35
1.6.6	<i>CWD strains</i>	36
1.6.7	<i>CWD in Europe</i>	40
1.6.7.1	<i>Surveillance prior to 2016</i>	40
1.6.7.2	<i>CWD cases in Europe</i>	41
1.6.7.3	<i>Transmission studies of European CWD</i>	43
1.6.8	<i>Future perspectives</i>	45
1.7	Thesis aims.....	47
2	Survey of the prion protein gene (PRNP) variation in British cervid species	48
2.1	Introduction.....	49
2.2	Materials and methods	54
2.2.1	<i>Contributions to the manuscript</i>	54
2.2.2	<i>Sample overview</i>	54
2.2.3	<i>Sample collection</i>	54
2.2.4	<i>DNA extraction</i>	55
2.2.5	<i>PRNP Polymerase Chain Reaction (PCR)</i>	55
2.2.6	<i>Primer design</i>	56
2.2.7	<i>Agarose gel electrophoresis</i>	57

2.2.8	<i>Activated charcoal purification of PCR products</i>	57
2.2.9	<i>Sequencing of PRNP</i>	57
Variation in the prion protein gene (PRNP) sequence of wild deer in Great Britain and mainland Europe.		
2.3	<i>Supplementary results</i>	58
2.3.1	<i>PRNP variation in exotic deer species in the UK</i>	58
2.4	<i>Discussion</i>	62
3	Developing an <i>in-vitro</i> conversion assay to assess the effects of PRNP genetic variation on chronic wasting disease susceptibility	68
3.1	<i>Introduction</i>	69
3.2	<i>Materials and methods</i>	73
3.2.1	<i>Molecular biology techniques</i>	73
3.2.1.1	<i>Vectors for the expression of cervid PrP in bacteria</i>	73
3.2.1.2	<i>Vectors for the expression of cervid PrP in mammalian cells</i>	73
3.2.1.3	<i>Mutagenesis reactions</i>	73
3.2.1.4	<i>Colony selection, bacterial growth, and plasmid preparation</i>	74
3.2.1.5	<i>Glycerol stocks</i>	75
3.2.1.6	<i>Bacterial transformation</i>	75
3.2.1.7	<i>Sequencing of plasmids</i>	75
3.2.2	<i>Production of recombinant cervid prion protein</i>	76
3.2.2.1	<i>Bacterial growth and induction of protein production</i>	76
3.2.2.2	<i>Bacterial lysis</i>	76
3.2.2.3	<i>Purification of recombinant protein</i>	77
3.2.3	<i>Fibrillisation assay</i>	77
3.2.3.1	<i>Maturation and PK digestion of fibrils</i>	78
3.2.4	<i>Subcloning of cervid PrP insert into pTRETight2 vector</i>	78
3.2.4.1	<i>Restriction enzyme digests</i>	78
3.2.4.2	<i>Agarose gel extraction and purification</i>	78
3.2.4.3	<i>Ligation of new vector</i>	78
3.2.4.4	<i>Diagnostic restriction digest and sequencing of new vectors</i>	79
3.2.5	<i>General cell culture methods</i>	79
3.2.5.1	<i>Cell lines and maintenance</i>	79
3.2.5.2	<i>Freezing of cells</i>	80
3.2.5.3	<i>Thawing and splitting of cell cultures</i>	80

3.2.5.4	<i>Lysis of cells</i>	80
3.2.5.5	<i>Total protein concentration estimations of cell lysates</i>	81
3.2.5.6	<i>PNGase treatment of cell lysates</i>	81
3.2.6	<i>Western blotting of cell lysates</i>	81
3.2.7	<i>Immunofluorescence microscopy</i>	82
3.2.8	<i>Transfection of RK13 cells</i>	83
3.2.8.1	<i>Transient expression: pcDNA3.1+ vector</i>	83
3.2.8.2	<i>Stable expression: pcDNA3.1+ vector</i>	83
3.2.8.3	<i>Transient expression: pTRE/pTetON vector</i>	84
3.2.8.4	<i>Stable expression: pTRE/pTetON vector</i>	84
3.2.9	<i>Protein misfolding cyclic amplification (PMCA)</i>	85
3.2.9.1	<i>Preparation of brain homogenates for use in PMCA</i>	85
3.2.9.2	<i>NaPTA precipitation and western blotting of PMCA seeds</i>	87
3.2.9.3	<i>Protein misfolding cyclic amplification (PMCA)</i>	87
3.2.9.4	<i>Western blotting of PMCA products</i>	89
3.3	<i>Results</i>	93
3.3.1	<i>Production of recombinant cervid prion protein and fibrillisation assay</i> 93	
3.3.2	<i>Expression of cervid PrP in cell culture</i>	95
3.3.3	<i>Mutagenesis for the 3F4 epitope in cervid PRNP</i>	95
3.3.4	<i>Transient transfection of RK13 cells with pcDNA3.1+_TPQ_3F4</i>	97
3.3.5	<i>Stable transfection of RK13 cells using pcDNA3.1+_TPQ_3F4</i>	102
3.3.6	<i>Construction of PrP-expressing vectors</i>	104
3.3.6.1	<i>Preparation of pTRE and pTet-ON plasmids</i>	104
3.3.6.2	<i>Subcloning of cervid PRNP into pTREtight2</i>	104
3.3.7	<i>Cervid PRNP expression in RK13 cells using pTRE and pTet-ON plasmids</i>	106
3.3.8	<i>Stable transfection, cloning and characterisation of stably transfected RK13 cell clones</i>	107
3.3.9	<i>Protein misfolding cyclic amplification (PMCA) using cell lysate as a substrate</i>	113
3.3.9.1	<i>Optimisation of PMCA using Rov9 lysate</i>	113
3.3.9.2	<i>Preparation of CWD brain material for use as PMCA seed</i>	115
3.3.9.3	<i>Testing of CWD seeds in PMCA</i>	116

3.3.9.4	<i>Optimisation of PMCA using cervid BSE.....</i>	117
3.3.9.5	<i>Cervid PRNP expressing cell lysate as substrate in PMCA.....</i>	118
3.4	<i>Discussion.....</i>	120
4	Primary transmission of CWD isolates from Norway and Canada in wild-type mice.....	126
4.1	<i>Introduction.....</i>	127
4.2	<i>Materials and methods.....</i>	132
4.2.1	<i>Norwegian and Canadian CWD isolates.....</i>	132
4.2.2	<i>Inoculation, clinical scoring, and tissue collection.....</i>	133
4.2.2.1	<i>CWD inocula preparation.....</i>	133
4.2.2.2	<i>Inoculation of experimental mice.....</i>	133
4.2.2.3	<i>Clinical monitoring and scoring.....</i>	134
4.2.2.4	<i>Incubation period or survival time.....</i>	134
4.2.2.5	<i>Post-mortem tissue collection.....</i>	135
4.2.2.6	<i>Analysis exclusion criteria.....</i>	135
4.2.3	<i>Histological analysis of mouse tissues.....</i>	135
4.2.3.1	<i>Fixation, embedding and sectioning of mouse brain for histological techniques.....</i>	135
4.2.3.2	<i>Vacuolation scoring and lesion profiles.....</i>	136
4.2.4	<i>Immunohistochemical analysis of brain tissues.....</i>	137
4.2.5	<i>Immunohistochemical analysis of spleen tissues.....</i>	138
4.2.6	<i>Thioflavin staining of amyloid.....</i>	139
4.2.7	<i>Biochemical analysis of mouse tissues.....</i>	139
4.2.7.1	<i>Homogenisation of mouse tissues.....</i>	139
4.2.7.2	<i>PK treatment and western blotting.....</i>	139
4.2.7.3	<i>NaPTA precipitation of spleen homogenates.....</i>	140
4.2.8	<i>RT-QuIC analysis.....</i>	141
4.3	<i>Results.....</i>	142
4.3.1	<i>Clinical features, incubation period and lesion profiling of CWD transmissions in wild-type mice.....</i>	142
4.3.2	<i>Survival time and clinical signs.....</i>	143
4.3.3	<i>Vacuolation/Lesion profiles.....</i>	146
4.3.4	<i>Immunohistochemical detection of prion protein deposition in the brains of wild-type mice.....</i>	148

4.3.5	<i>Classification of PrP^D distribution patterns and staining types.....</i>	<i>148</i>
4.3.6	<i>Transmission of Canadian CWD isolates.....</i>	<i>152</i>
4.3.6.1	<i>PrP deposition patterns vary between VM, C57BL/6 and RIII mice inoculated with Can-WT isolate</i>	<i>153</i>
4.3.7	<i>Transmission of Norwegian CWD isolates</i>	<i>156</i>
4.3.7.1	<i>PrP^D deposition in Nor-R inoculated mice</i>	<i>156</i>
4.3.8	<i>Alternative staining in CWD inoculated mice.....</i>	<i>159</i>
4.3.8.1	<i>Haemosiderin.....</i>	<i>159</i>
4.3.8.2	<i>Unusual staining type</i>	<i>160</i>
4.3.9	<i>Biochemical characterisation of CWD transmission in the brains of wild-type mice</i>	<i>160</i>
4.3.9.1	<i>Optimisation of western blotting</i>	<i>161</i>
4.3.9.2	<i>Western blotting of Canadian transmissions.....</i>	<i>162</i>
4.3.9.3	<i>Western blotting of Norwegian transmissions.....</i>	<i>162</i>
4.3.10	<i>Rt-QuIC amplification for the detection of CWD in mouse brain homogenates.....</i>	<i>164</i>
4.3.11	<i>Is Lymphoid replication an important feature of primary passage of CWD in wild-type mice?</i>	<i>168</i>
4.3.11.1	<i>Immunohistochemical detection of PrP^D in the spleens of wild-type mice inoculated with CWD.....</i>	<i>169</i>
4.3.11.2	<i>NaPTA precipitation of PrP^{Sc} from wild-type mice spleens.....</i>	<i>171</i>
4.3.11.3	<i>RT-QuIC amplification of spleen samples from wild-type mice</i>	<i>173</i>
4.4	<i>Discussion.....</i>	<i>178</i>
5	Final discussion	187
6	References	197
7	Appendix.....	234

Figure contents

Figure 1-1: Structure of the mammalian prion protein.....	
Figure 1-2: Simplified schematic representation of in-vitro assays used for the amplification of PrP ^{Sc}	
Figure 1-3: Map showing location of chronic wasting disease cases in Scandinavia.....	
Figure 2-1: Distribution of British deer species in the 2016 Deer Distribution survey by the British Deer Society.	
Figure 2-2: Non-synonymous variation in the PRNP ORF of British wild and exotic cervid species.....	
Figure 3-1: Schematic representation of approaches to produce recombinant PrP ^C for use in in-vitro conversion assays.....	
Figure 3-2: Purification of cervid prion protein (QGAS) by (A) NiIMAC and (B) reverse phase HPLC.	
Figure 3-3: Proof of concept and initial results of fibrillation kinetics for two variants (a) GMSE (Elk 132M) and (b) GLSE (Elk 132L).	
Figure 3-4: Sequencing chromatograms to show mutagenesis for the 3F4 epitope in pcDNA3.1+ vector with cervid TPQ PRNP gene	
Figure 3-5: Expression of cervid PrP ^C in RK13 cells after transient transfection with the pcDNA3.1+_TPQ_3F4 vector.	
Figure 3-6: Comparison of PrP ^C expression between cell lysates (RK13_3F4+ and Rov9) and red deer brain homogenates of 226EE and QQ genotypes.....	
Figure 3-7: Immunofluorescence of PrP ^C expression using ROS-BC6 in RK13 cells transiently transfected with pcDNA3.1+_TPQ_3F4.....	
Figure 3-8: Immunofluorescence of PrP ^C expression using ROS-BC6 in RK13 cells stably transfected with pcDNA3.1+_TPQ_3F4	
Figure 3-9: Restriction enzyme digests of pTet-on and pTREtight2 plasmids to confirm plasmid identity and subcloning of cervid PRNP.....	
Figure 3-10: Transient transfection of RK13 cells with pTREtight2_cPrP_3F4_TPQ	
Figure 3-11: Immunofluorescence analysis of pools of stably transfected cells comparing different transfection protocols.....	
Figure 3-12: Subcloning of RK13 cells transfected with pTREtight2_cPrP_3F4_TPQ. Immunofluorescence using the ROS-BC6 antibody.	

Figure 3-13: Further subcloning of transfected cell clones (clone 12).....	
Figure 3-14: Further analysis of subclone 5 by immunofluorescence and western blotting.....	
Figure 3-15: Amplification of natural scrapie with Rov9 lysate (top) and tg338 (ovine VRQ) brain homogenate (bottom) as substrate after one round of PMCA.....	
Figure 3-16: Amplification of natural scrapie with Rov9 lysate as substrate after one round of PMCA.....	
Figure 3-17: Western blot analysis of PrP ^{Sc} in brains of experimental WTD, for use as seeds in PMCA.....	
Figure 3-18: Amplification of cervid BSE using TgshpXI mouse (expressing ovine ARQ PrP ^C) (top) or cervid (bottom) brain homogenate as substrate.	
Figure 4-1: Location of the four rostro-caudal levels taken to produce coronal sections in the fixed mouse brain. Arrows indicate the face-up direction of the section. (Image from SOP – Roslin Institute)	
Figure 4-2: Schematic diagram of the coronal sections and scoring regions used in lesion profiling.....	
Figure 4-3: Lesion profiles in VM (A) and C57BL/6 (B) mice after primary passage with Can-WT isolate. Data points represent the mean±SEM of ≥5 mice per group.....	
Figure 4-4: Histopathological and immunohistochemical analysis of a VM mouse inoculated with the Can-WT isolate.....	
Figure 4-5: PrP ^D distribution patterns in wild-type mice inoculated with CWD.....	
Figure 4-6: Plaque characterisation in a C57BL/6 mouse inoculated with Can-WT isolate (M00132817).	
Figure 4-7: Comparison of PrP ^D deposition in the thalamus and cerebellum of wild-type mice inoculated with Can-WT isolate.	
Figure 4-8: PrP ^D deposition using 6H4 antibody in the brain of a C57BL/6 mouse (M00111889) inoculated with Nor-R isolate.	
Figure 4-9: PrPD deposition using 6H4 antibody in the brain of a C57BL/6 mouse (M00111925) inoculated with Norwegian reindeer CWD isolate.....	
Figure 4-10: Haemosiderin deposition following a needle-track in the brain of a Nor-M mouse found dead at 138dpi.	
Figure 4-11: Unusual staining observed upon transmission of CWD isolates in wild-type mice.....	

Figure 4-12: Optimisation of western blot protocol.....	
Figure 4-13: Western blot analysis of PrP ^{Sc} in the brains of three lines of wild-type mice inoculated with Can-WT isolate.	
Figure 4-14: Western blot for the detection of PrP ^{Sc} in the brains of RIII mice inoculated with (A) Nor-M (lanes 1-4) and Nor-R (lanes 5-10) isolates and (B) a C57/Bl6 mouse inoculated with Nor-M with detectable PrP ^{Sc} after image enhancement.....	
Figure 4-15: RT-QuIC amplification of CWD using bank vole recombinant substrate.....	
Figure 4-16: Amplification of a panel of mouse brain homogenates from Nor-M, Nor-R and Can-WT transmissions.	
Figure 4-17: Immunohistochemical detection of PrP ^D in the spleens of wild-type mice using BH1 antibody.	
Figure 4-18: Optimisation of NaPTA protocol for precipitation of PrP ^{Sc} in wild-type mouse spleens.....	
Figure 4-19: NaPTA precipitation and western blotting of wild-type mouse spleens.	
Figure 4-20: RT-QuIC amplification spleen samples before (A) and after NaPTA precipitation (B).	
Figure 4-21: RT-QuIC optimisation and testing using NaPTA precipitated spleen samples.....	
Figure 4-22: RT-QuIC amplification of NaPTA precipitated spleen samples.....	
Figure 7-1: Representative PrP ^D deposition in the brain of a VM mouse inoculated with Can-WT isolate.	
Figure 7-2: Representative PrP ^D deposition in the brain of a C57BL/6 mouse inoculated with Can-WT isolate.....	
Figure 7-3: Representative PrP ^D deposition in the brain of an RIII mouse inoculated with Can-WT isolate.....	

Table contents

Table 1-1: Increasing recognition of animal prion diseases.....	5
Table 1-2: Overview of European CWD cases	45
Table 2-1: Standard PCR reaction for amplification of PRNP	
Table 2-2: Primers for the open reading frame (ORF) of cervid PRNP.....	
Table 2-3: Primer pairs effective for amplification of cervid PRNP by species.....	
Table 2-4: Sequencing reaction mix using the BigDye Terminator v3.1 Cycle Sequencing Kit.....	
Table 2-5: Amino acid variation in the PRNP sequence of exotic cervid species.	
Table 3-1: Site-directed mutagenesis reactions performed to generate British PRNP variants and the 3F4 epitope.....	74
Table 3-2: Sequencing reaction mix using the BigDye Termination v3.1 cycle sequencing kit.....	76
Table 3-3: Brain homogenates used as PrP ^C substrate or PrP ^{Sc} seed for PMCA experiments.	86
Table 3-4: Comparison of PMCA protocols tested for the optimisation of PMCA using cell lysate.	88
Table 3-5: Transient and stable transfection conditions attempted with pcDNA3.1+_TPQ_3F4 vector in RK13 cells.	90
Table 3-6: Transient and stable transfection conditions attempted with pTREtight2_cPRP_TPQ_3F4 and pTet-ON vectors in RK13 cells.	91
Table 3-7: Species comparison of the 3F4 epitope.....	
Table 4-1: Geographical location, species, origin, age and PRNP genotype of the CWD affected cervids studied by transmission to wild-type mice.....	133
Table 4-2: Clinical scoring system for TSE infected mice.....	134
Table 4-3: Criteria for vacuolation scoring in mice.....	
Table 4-4: Primary transmission of Canadian wild mule deer (Can-M) and experimental white-tailed deer (Can-WT) to wild-type mice.....	
Table 4-5: Primary transmission of Norwegian reindeer (Nor-R) and moose (Nor-M) isolates to wild-type mice.....	

Table 4-6: Overview of IHC positive mice inoculated with Can-WT isolate.	
Table 4-7: IHC positive mice from Norwegian transmission experiments	156
Table 4-8: RT-QuIC analysis of mouse brain homogenates from Can-EM, Nor-R and Nor-M transmissions.....	
Table 4-9: RT-QuIC analysis of NaPTA precipitated spleen samples from CWD transmissions in wild-type mice.....	
Table 7-1: Overview of transmission results for Norwegian CWD isolates in wild-type mice	238
Table 7-2: Overview of transmission results for Canadian CWD isolates in wild-type mice	242

Abbreviations

BDS	British Deer Society
BSE	Bovine spongiform encephalopathy
CL2/3	Containment level 2/3 laboratories
CNS	Central nervous system
CSF	Cerebrospinal fluid
CWD	Chronic wasting disease
dpi	Days post inoculation
EFSA	European Food Standards Agency
EUE	Exotic Ungulate Encephalopathy
FDC	Follicular dendritic cells
FSE	Feline Spongiform encephalopathy
GL	Granular layer of cerebellum
GPI	glycosyl-phosphatidyl-inositol
HPLC	High-performance liquid chromatography
IHC	Immunohistochemistry
IMAC	Immobilised metal affinity chromatography
MD	Molecular dynamics
ML	Molecular layer of cerebellum
MW	Molecular weight
NMR	Nuclear Magnetic Resonance
NS	Natural (classical) scrapie
ORF	Open reading frame
PCR	Polymerase chain reaction
PET	Paraffin embedded tissue
PK	Proteinase K
PMCA	Protein Misfolding Cyclic Amplification
PRNP	Prion protein gene
PrP ^C	Normal cellular form of the prion protein
PrP ^D	Disease associated abnormal prion protein deposition detected by IHC

PrP ^{Sc}	PK resistant, misfolded, disease associated form of prion protein
PrP ^{sen}	Protease sensitive prion protein
RAMALT	Rectoanal musosa associated lymphoid tissue
RT-QuIC	Real-time Quaking Induced Conversion
sCJD	Sporadic Creutzfeldt Jakob disease
SEM	Standard error of the mean
senPrP ^{Sc}	Proteinase K sensitive isoform of PrP ^{Sc}
SNP	Single nucleotide polymorphism
TSE	Transmissible spongiform encephalopathy
vCJD	Variant Creutzfeldt Jakob disease
WB	Western blot
wt	Wild-type
WTD	White-tailed deer

1 Introduction

1.1 An introduction to transmissible spongiform encephalopathies

1.1.1 *General introduction*

The transmissible spongiform encephalopathies (TSE), or prion diseases, are a diverse and enigmatic group of neurodegenerative disorders affecting humans and animals. This family of diseases includes scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) of cattle, chronic wasting disease (CWD) of deer, and a range of human diseases including sporadic (sCJD) and variant (vCJD) Creutzfeldt Jakob disease. All prion diseases are transmissible, however only some are classically infectious and able to spread from one host to another in the natural environment, such as scrapie and CWD. Other prion diseases may arise sporadically in the host, be hereditary due to mutations in the prion protein gene, or spread via iatrogenic routes such as contaminated blood, tissue or surgical instruments. The molecular event at the centre of all TSE disease is the conversion of the host normal prion protein termed 'PrP^C' to a protease resistant misfolded form 'PrP^{Sc}'. This misfolded protein is prone to aggregation and accumulates principally in the central and peripheral nervous system and lymphoreticular tissues. Prion diseases are characterised histologically by vacuolation, gliosis and neuronal loss, and immunohistochemically by the accumulation of PrP^{Sc}. Though the presence, tissue distribution and protease resistance of PrP^{Sc} may vary depending on prion disease (see section 1.2.1), it is widely regarded as a hallmark of TSE diseases, and detection of PrP^{Sc} forms the basis of diagnostic tests.

1.1.2 *The history of prion diseases*

Early reports of a scratching disease in sheep began as early as the 18th century, with one report written in Germany in 1759 accurately describing the clinical characteristics and contagious nature of scrapie [1]. In the 19th century scientific investigations into the disease started, with neuropathological examination revealing the characteristic neuronal vacuolation in the absence of any obvious

pathogen [2]. Early attempts to transmit scrapie by inoculation failed due to lack of recognition of the extremely long incubation periods, and was not successful until 1936 [3]. The first human prion disease, sporadic CJD (sCJD), was described by neurologists Creutzfeldt and Jakob in 1920 [4]. Kuru, a similar neurodegenerative disease, was described in 1957 in the Fore linguistic group of Papua New Guinea [5]. It is likely that the kuru epidemic originated in a case of sCJD that was transmitted during traditional funeral rituals involving the consumption of central nervous system (CNS) tissues of deceased relatives [6]. In 1959 scrapie, CJD and kuru were defined for the first time as distinct forms of the same neuropathy [7, 8]. Reminiscent of kuru in humans, the outbreak of BSE in the UK in the 1980's is believed to have originated either from scrapie in sheep or from a spontaneous prion disease in cattle, which amplified in the cattle population due to the recycling of carcasses from infected animals to produce meat and bone meal for inclusion into animal feeds. BSE grew to epidemic status in the UK and had profound effects on the beef industry in the UK and other countries, resulting in increased research efforts to better understand TSEs. The identification of a new distinct form of Creutzfeldt Jacob disease (variant, vCJD) in humans in the UK was later linked to the consumption of BSE-infected meat [9, 10].

1.1.3 New and emerging threats

Whilst the BSE epidemic was brought under control by bans on feeding meat and bone meal to livestock, surveillance and removal of specified risk materials from the human food chain, a significant proportion of the UK population was exposed to contaminated meat [11]. In the UK vCJD has caused 178 deaths to date, peaking at 28 in the year 2000, however in recent years only one death has been recorded in 2016 [12]. The possibility of sub-clinically infected carriers has been identified in retrospective analysis of archived appendix and tonsil samples in the UK [13–15], and investigations are currently focused on diagnostics for identifying individuals whom may be able to transmit disease through blood or organ donation [16]. Prion diseases continue to be recognised,

either by chance, as demonstrated by identification of chronic wasting disease in a Norwegian reindeer in 2016 [17], or due to recognition of a previously known neurological disease as a TSE, as was the case for camel prion disease in 2018 [18].

1.1.4 Atypical prion diseases

The increased awareness and active surveillance for TSEs occurring in response to the BSE epidemic facilitated the discovery of so-called atypical prion diseases in sheep (atypical/Nor98 scrapie) and in cattle (H- and L-type BSE), reviewed in [19]. These diseases are distinct from classical prion diseases in many of their characteristics, including neuropathology and apparent lack of lymphotropism. The age-distribution of affected animals is also an important factor, with the typical age of classical BSE cases being three to five years, whereas atypical BSE cases usually occur in animals over eight years old. Whilst human prion disease cases are predominantly of sporadic origin, sporadic prion diseases in animals are yet to be formally identified. This may be due to the low incidence of disease, unusual or absent clinical signs, specific diagnostics necessary to distinguish atypical from classical disease, and to the old age-distribution of atypical cases, with elderly animals not often present in production animal populations. Thus, identification relies upon either large scale screening programmes with numbers sufficient to capture these rare cases, or general awareness of prion diseases in veterinarians that promote specific testing. An example of the latter is the identification of an H-type BSE case in a 19 year-old miniature zebu (*Bos indicus*) in a zoological park, which was exhibiting neurological clinical signs [20].

Table 1-1: Increasing recognition of animal prion diseases

Disease		Date first identified	Country identified	Host species	Origin
Scrapie	Classical scrapie	1730	UK	Sheep	Unknown
	Atypical scrapie NOR98	1998 ¹	Norway	Goats Sheep	Unknown
Transmissible mink encephalopathy	TME	1947	USA	Mink	Unknown
Chronic wasting disease	CWD	1967 ²	USA	Deer, elk, reindeer, moose	Unknown
	'Novel' CWD	2016	Norway	Moose, red deer	Unknown
Bovine spongiform encephalopathy	Classical BSE	1985	UK	Cattle	Unknown
	Atypical BSE	2004	France /Italy	Cattle	Unknown
Feline spongiform encephalopathy	FSE	1990	UK	Cats	BSE-contaminated feed
Exotic ungulate encephalopathy	EUE	1986	UK	Ungulates	BSE-contaminated feed
Goat BSE		2005	France	Goats	Unknown
Camel prion disease	CPD	2018	Algeria	Camels	Unknown

¹Restrospective analysis has identified atypical scrapie in 1972

²Not recognised as a TSE until 1978

1.2 The nature of the TSE agent

The early hypotheses on the aetiology of scrapie were of a “slow virus” due to the recognition of its infectious nature and long incubation period [7, 21]. However, the first evidence against this theory was produced as early as 1944, during the vaccination of sheep against louping ill [22]. The vaccine contained preparations of brain and spleen tissue treated with formalin, which successfully inactivated the louping ill virus, but not the scrapie agent that was also present, leading to a large number of scrapie cases in the vaccinated animals. Further experiments showed that scrapie infectivity is highly resistant to ionising and UV radiation, extreme heat, high pressure, nucleases and other compounds known to inactivate viruses and bacteria [23–28]. Some of these treatments were known to destroy nucleic acids, generating speculation of a proteinaceous scrapie agent, first by Griffith in 1967 [29], and later by Prusiner, in work for which he would win a Nobel prize [28].

1.2.1 *The prion hypothesis*

The ‘protein only’ or prion (*proteinaceous infectious agent*) hypothesis states that the misfolded form of the prion protein is a novel infectious agent devoid of a nucleic acid component [28]. This misfolded and protease resistant protein, “PrP^{Sc}”, can self-template and catalyse the conversion of the cellular form of the protein “PrP^C”. Accumulation of PrP^{Sc} in the central nervous and/or lymphoreticular systems is a hallmark of TSE disease. The unusual resistance of the agent to chemical and physical inactivation methods (see above), in combination with the reduction in infectivity by anti-PrP antibodies and compounds that destroy protein structure [30], has provided convincing evidence of the importance of the protein in disease. Whilst the prion hypothesis is now widely accepted by the scientific community [31], some scientists still pursue evidence of a slow viral, viroid or bacterial aetiology [32, 33]. Increasing evidence of bacteria and viruses able to survive extreme environmental conditions, including resistance to autoclaving, boiling, formalin

treatment and gamma irradiation, opens the possibility of the involvement of these agents. However, despite numerous attempts, the evidence for viral or bacterial aetiology in prion diseases remains unconvincing. Described as the 'holy grail' of prion research, attempts to generate prions *in-vitro* that are infectious *in-vivo* provides compelling evidence of the prion hypothesis. Early attempts included partially purified PrP^{Sc} from brain homogenates that could be argued to contain another infectious agent. Misfolded purified recombinant PrP has since been shown to induce disease in hosts overexpressing PrP^C, and also in wild-type animals [31, 34–39]. Recent studies have highlighted the importance of co-factors in the infectivity of recombinant prions produced *in-vitro* [40, 41].

1.2.2 Isoforms of the prion protein

The cellular form of the prion protein is sensitive to proteases such as proteinase K (PK). Host expression of PrP^C is a pre-requisite for disease, as demonstrated by the resistance of goats and transgenic mice devoid of PrP^C expression to scrapie [42, 43]. Induced ablation of PrP^C expression in prion-infected mice halts the development of prion disease and rescues early neuropathological changes, despite continuing accumulation of PrP^{Sc} in the brain [44]. Isolation of pathogenic misfolded and protease-resistant PrP^{Sc} from infectious material and evidence that concentration of PrP^{Sc} is proportional to infectivity titre provide further support for the prion hypothesis [28, 45]. However, the dissociation of PrP^{Sc} and infectivity has also been reported, with both infectivity in the absence of detectable PrP^{Sc}, and PrP^{Sc} in the absence of infectivity [46–48]. The former may be explained by the presence of protease sensitive forms of PrP^{Sc} (senPrP^{Sc}) that have been identified in both animal and human TSE cases, whilst the latter may be explained by PrP^{Sc} aggregation states associated with differences in infectivity [49]. Importantly, altered PrP expression and associated abnormal PrP labelling has been reported in sheep with a variety of non-prion neurological disorders [50]. Additionally, altered distribution, aggregation and increased insolubility of the cellular prion protein

has been reported following intracranial inoculation of mice, together suggesting that damage to the brain may be a trigger for the refolding of PrP into pathogenic isoforms even in the absence of prion infection [51].

1.2.3 Prions and proteinaceous nucleating particles

Attention has recently been focused on the shared characteristics of prions and other proteins, described as “prion-like”, “prionoid”, and most recently “proteinaceous nucleating particles”. Early corroboration of the prion hypothesis as a biological process was provided by the discovery of proteins in yeast that behave as prions [52]. The conceptual framework of self-propagating infectious proteins could then not only be applied to prion diseases, but also to other diseases involving pathological protein aggregation, especially neurodegenerative disorders. There is mounting evidence that at least some of the properties of misfolded prion protein are shared with other proteins associated with diseases such as Alzheimers (amyloid-beta [A β] and tau), Parkinson’s (α -synuclein), frontotemporal dementia (TDP43, tau, FUS), and motor neurone disease (TDP43). These shared characteristics include self-propagation through seeding activity, as well as the potential (of A β) to transmit between humans [53–55].

1.3 The prion protein in health and disease

Whilst there are overarching mechanisms common to neurodegenerative diseases caused by misfolding proteins, there are also disease-specific mechanisms determined by the cell biology and biochemistry of the protein involved [56]. In the case of TSEs the underlying trigger may be genetic, sporadic or of infectious origin, causing the conversion of normal cellular protein (PrP^C) to a conformationally altered (misfolded) isoform (PrP^{Sc}) which is protease resistant, rich in β -sheets and prone to aggregation, leading to accumulation in the CNS and characteristic neurodegeneration.

1.3.1 Structure and cleavage of PrP^C

The cellular prion protein (PrP^C) is a cell surface glycoprotein mainly located in lipid rafts, attached to the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The protein is expressed in a wide variety of tissue types with varying expression levels, with highest expression in the central and peripheral nervous systems. PrP^C is initially expressed as a precursor protein of approximately 250 amino acids and follows a typical

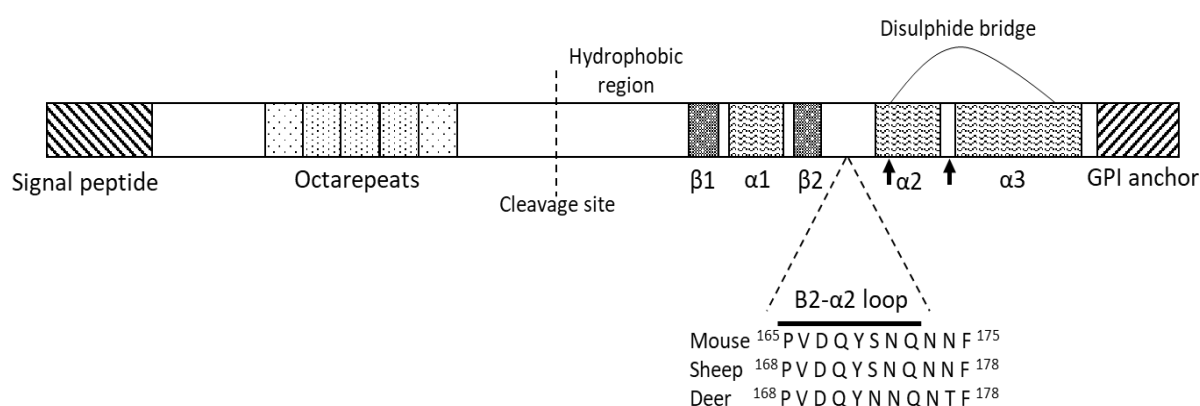


Figure 1-1: Structure of the mammalian prion protein. Arrows show sites of N-linked glycosylation.

journey through the rough endoplasmic reticulum and Golgi body then to the cell membrane via the attachment of a glycosyl-phosphatidyl-inositol (GPI) anchor. Post-translational modifications of the protein include cleavage of both the N terminal and C terminal signal peptides to produce a mature protein of 208-210 amino acids (residues 23 to 230-232 of the precursor protein depending on species)[57]. PrP^C features an unstructured flexible N-terminal domain, which has a stretch of octapeptide repeats, and a structured, globular C-terminal domain containing three α -helices and two β -sheets (Figure 1-1). Within the C-terminal domain there are two sites of N-linked glycosylation, which may be variably glycosylated to produce the characteristic three banding pattern of PrP^C on western blot, (di-, mono- and un-glycosylated). Cleavage of the prion protein may produce smaller bands: α -cleavage is considered to be part of the normal proteolytic processing and produces anchored C1 and free N1 proteins; β -cleavage occurs at low levels in response to stress, and produces

the slightly longer anchored C2, and free N2 proteins [58]. Whilst the majority of PrP^C is presented at the cell surface and undergoes cycles of internalisation via endosomes, some may be released or shed from the cell surface following the action of phospholipases on the GPI anchor or ADAM proteases on the C-terminus [59, 60].

1.3.2 Function of PrP^C

A key question remaining in prion research is whether disease pathogenesis is due to the toxic effects of PrP^{Sc} or due to the loss of function of PrP^C. Despite extensive study of the cellular prion protein and proposal of many possible functions, reports are sometimes contradictory and the specific functions of PrP^C remain elusive. However, some of the most suggested roles include neuroprotective and cell-signalling functions, association with regulation of sleep, and most recently roles in myelin maintenance [59, 61, 62]. The high level of conservation of PrP sequence in mammalian species is suggestive of a vital function [63, 64]. Surprisingly, the first lines of PrP^C-knockout mice (PrP^{0/0}) showed no obvious phenotypes suggestive of loss of function [65]. Since then more knockout mice have been generated with various phenotypes, however these appear to be due to unintentional downstream effects of generating transgenics, rather than loss of PrP^C [66–68]. Norwegian goats naturally devoid of prion protein (PrP^C-null) were likewise healthy [69].

1.3.3 Conversion of PrP^C to PrP^{Sc}

The conversion of PrP^C to disease-associated, infectious, misfolded PrP^{Sc} is central to the protein-only hypothesis and our understanding of prion diseases. The conversion of PrP^C involves a purely structural conformational change and involves enrichment for β -sheet structures which introduce a marked propensity to aggregate. Two molecular pathways have been hypothesised to explain how this change may occur. The first, sometimes known as the template assisted conversion model, states that the interconversion of PrP^C and PrP^{Sc} is separated by an energy barrier (and therefore cannot occur spontaneously).

The interaction of PrP^C and PrP^{Sc} (with or without an additional co-factor producing an intermediate PrP* conformation) produces a heterodimer which catalyses the conversion. The subsequent addition of PrP^C monomers allows formation of a fibril. Alternatively, PrP^C and PrP^{Sc} may exist in equilibrium, that in a healthy brain is heavily in favour of PrP^C formation. PrP^{Sc} gains stability during interaction with another PrP^{Sc} molecule and can disrupt the equilibrium to promote formation of further PrP^{Sc} molecules. This 'seeding/nucleation' model supports the idea of spontaneous conversion of PrP^{Sc}, with both genetic mutation or co-factors able to disrupt the equilibrium [59].

1.3.4 Detection of PrP^{Sc}

The post-mortem neuropathological examination of brain tissue from humans or animals remains the gold-standard for the diagnosis of TSEs. As clinical signs and vacuolation occur relatively late in the disease process, the detection of PrP^{Sc} accumulation is a useful diagnostic marker. PrP^{Sc} may be detected using immunohistochemistry (IHC) on tissue sections or western blotting of tissue homogenates, using specific anti-prion protein antibodies.

Immunohistochemistry allows for analysis of PrP^{Sc} distribution and staining patterns, which can assist in the differentiation of different prion diseases and prion strains. Western immunoblotting (WB) provides a valuable alternative to IHC if the condition of the brain does not allow for histopathological examination (which may often be the case for autolytic wild animal carcasses), is more rapid than IHC, and does not require specialised histopathological interpretation. Tissue homogenisation, proteinase-K digestion (to remove PrP^C) and western blotting allows visualisation of PrP^{Sc} specific banding patterns between 17kDa and 30kDa, representing diglycosylated, monoglycosylated and unglycosylated PrP^{Sc}. Isolates may be differentiated by variation in the site of PK digestion resulting in the removal of PrP epitopes, producing differential binding by specific antibodies as well as different banding patterns (ratios of the three glycoforms and molecular weight of the fragments). This analysis forms the basis of phenotypic typing in ruminants and humans (see section 1.5), for

example between classical, atypical and CH1641 scrapie in sheep and goats [70]. Rapid tests have also been developed for TSE detection. Two of the most commonly used kits validated for testing of sheep and cattle brains rely on the detection of PrP^{Sc} by enzyme immunoassay reactions [71]; the first is a sandwich ELISA for detection of PrP^{Sc} following proteinase-K digestion (TeSeE, BioRad®), the second uses a PrP^{Sc} specific ligand on a capture plate (HerdCheck IDEXX®). Confirmatory tests for any positive results are undertaken using non-rapid methods as described above.

1.3.5 *In-vitro amplification of PrP^{Sc}*

Prion protein amplification techniques harness the ability of PrP^{Sc} to self-propagate, so that small amounts of PrP^{Sc} present in tissues and biological fluids may be amplified to detectable levels. As well as forming the basis of extremely sensitive diagnostic techniques, these amplification assays have provided support for the prion hypothesis and aided our understanding of prion biology. The misfolding in these assays is driven by the addition of a 'seed', which is a small volume of PrP^{Sc} usually in the form of a tissue extract or body fluid from an infected animal. The cell-free conversion assay was developed during the 1990's and is the predecessor to the amplification techniques now routinely used in prion research [72, 73]. These initial studies demonstrated that PrP^{Sc} purified from a scrapie infected hamster brain could be used to induce conversion of hamster PrP^C derived from tissue culture to protease-resistant forms similar to PrP^{Sc}, although this process was inefficient [72]. Studies using this assay provided evidence in support of the prion hypothesis (see section 1.2.1) and recapitulated some aspects of disease such as genetic modulation of susceptibility [74, 75] and species barriers [76]. More efficient methods for the *in-vitro* conversion of PrP^C have been developed in the last 15 years, which are described below and in Figure 1-2.

1.3.5.1 Protein misfolding cyclic amplification (PMCA)

PMCA was first described in 2001 [77] and generally employs brain homogenate [78] to provide a PrP^C rich substrate for conversion when seeded with a variety of different biological tissues or fluids containing PrP^{Sc}. However, the use of animal derived material, as well as the often limited availability of brain homogenate from certain species or genotypes, has driven the use of alternative substrates. Extracts from mammalian cell lines overexpressing PrP [79, 80], or recombinant PrP [81] have both been used successfully as PMCA substrate. However, brain homogenates contain a variety of co-factors for conversion, such as glycosaminoglycans, lipids and nucleic acids, which together with high concentrations with PrP^C may play a role in efficient *in-vitro* amplification [40, 82, 83]. The PMCA reaction consists of cycles of sonication (to break up aggregates and improve seeding) and incubation (to allow conversion/formation of aggregates) (Figure 1-2). To further increase sensitivity, serial rounds of PMCA may be performed, where a small amount of PMCA product is used to seed a fresh reaction [84, 85]. The addition of various compounds such as dextran sulphate [86] or beads (ceramic or Teflon®) has also been shown to improve PMCA efficiency [79, 87]. Western blotting of the amplified material allows detection as well as identification of PrP^{res} isotypes, which may be important for determining the nature/strain of the TSE agent in the seed (section 1.5). PMCA is regarded as a more physiologically relevant assay than RT-QuIC (see below), as the products generated retain the biochemical and structural properties of the seeding material, and are infectious in bio-assay [88–91].

1.3.5.2 Real-time Quaking Induced Conversion (RT-QuIC)

The Real-time Quaking Induced Conversion assay was developed following earlier similar assays called the amyloid seeding assay (ASA) or fibrillisation assays. These assays use purified recombinant PrP^C (recPrP) with (RT-QuIC, ASA) or without (fibrillisation) a PrP^{Sc} seed from an infected animal. Unlike PMCA, RT-QuIC combines protein amplification with an integral detection

system [92, 93]. Protein aggregates are formed and broken up by intermittent shaking (rather than sonication) and detected by the fluorescence of thioflavin-T when bound to amyloid aggregates. Fluorescence is measured on a plate reader and after an initial lag phase, a rapid increase in fluorescent signal is produced in positive samples. The kinetics of fibril formation are monitored in real time and may be analysed for parameters such as lag time and amyloid formation rate. RT-QuIC analysis is highly sensitive and specific for the detection of sCJD in human cerebrospinal fluid, and this test is now part of the official diagnostic criteria [94]. A variety of recombinant substrates including truncated and full length prion proteins have been used in RT-QuIC, however the most universal appears to be the bank vole prion protein [95, 96]. To date,

products produced by RT-QuIC amplification do not appear to be infectious *in-vivo* [88].

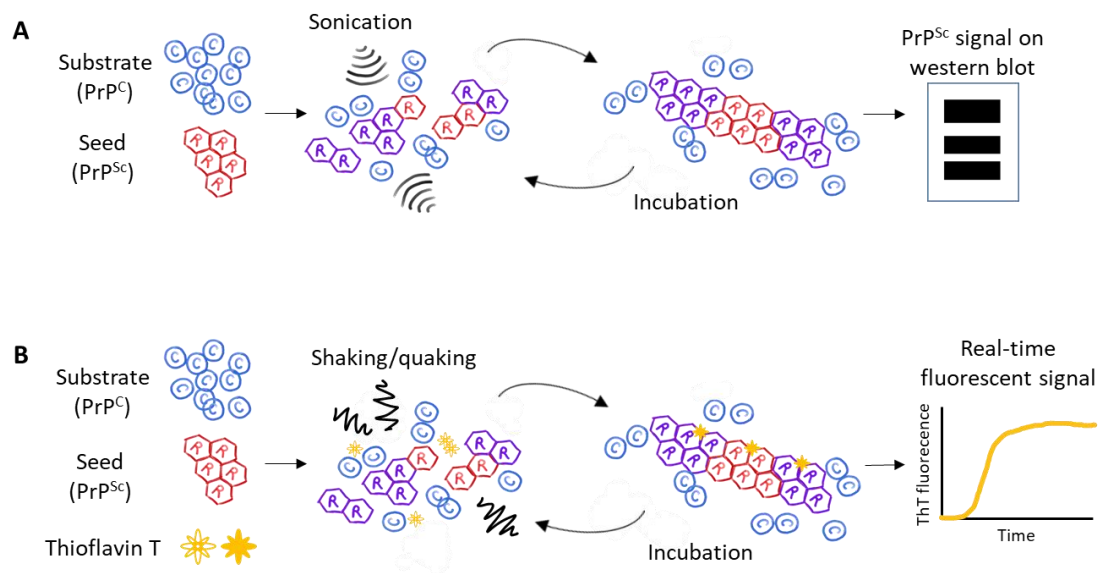


Figure 1-2: Simplified schematic representation of in-vitro assays used for the amplification of PrP^{Sc}.

- A) Protein misfolding cyclic amplification (PMCA). Schematic shows incubation and sonication in a single cycle of PMCA amplification which would be repeated a number of times (e.g. 96) for a single round.
- B) Real-time quaking induced conversion (RT-QuIC). Schematic shows generation and elongation of amyloid fibrils, and integration of Thioflavin T (ThT) which produces a fluorescent signal when bound to amyloid.

Blue: PrP^C substrate, red: PrP^{Sc} seed, purple: new misfolded PrP^{Sc}.

1.3.6 Pathogenesis of prion disease

The transmission and pathogenesis of prion diseases vary depending on the agent. This section will focus on CWD in deer and scrapie in sheep, which share many similarities in their pathogenesis including their pathology, incubation period (18 months-5 years) and transmission routes (direct contact or via environmental fomites) [97]. Once peripherally acquired prions enter the host, usually by the oral route, infection takes place via the accumulation of PrP^{Sc} in secondary lymphoid organs. Oral infection of mice and sheep with scrapie, and deer with CWD, results in accumulation first in the gut-associated lymphoid tissues (GALT) such as the Peyer's patches [98–102]. Much of our

understanding of prion pathogenesis has been gained through the study of prion infection (usually scrapie strains) in experimental mice, and is reviewed in [103]. In short, orally acquired prions cross the gut epithelia via specialised phagocytic cells (M cells) associated with Peyer's patches, or directly through enterocytes. Dendritic cells (DCs) located just below the intestinal epithelium are ideally placed to acquire and transport prions to lymphoid tissues, where prions replicate and accumulate mostly on follicular dendritic cells (FDCs). The accumulation of prions in the lymphoid tissues is not essential for neuroinvasion [104], however absence of the spleen, Peyer's patches or draining lymph node results in delayed neuroinvasion when infecting intraperitoneally, orally or via skin lesions respectively [105–107]. Lymphoid tropism of some prion strains is also important in cross-species transmission [108]. Neuroinvasion occurs along sympathetic and parasympathetic nerves, though the mechanisms involved in the passage of prions from the FDC to nerves are unclear, and may involve either DCs or exosomes [103]. Once established in the CNS, the earliest observed changes include synaptic loss, the accumulation of misfolded PrP, and reactive gliosis. Neuronal loss is identified relatively late in the disease process and is often seen in combination with clinical signs. The catalyst of these observable changes is still under debate, and it is unclear if misfolded prion protein is directly neurotoxic, if the loss of normal PrP^C function is detrimental, if the imbalance of neuronal homeostasis due to PrP^{Sc} replication plays a role, or if there is a combination of these factors [109, 110].

1.4 The prion protein gene and TSE susceptibility

1.4.1 *The prion protein gene*

The prion protein gene (*PRNP*) plays a key role in all TSE diseases. Early in the investigation of these diseases, both the sequence of *PRNP* [111] and the level of expression [43, 112] were linked to changes in TSE disease progression and susceptibility. *PRNP* is remarkably well conserved in mammals, and two homologs of the prion protein with similar structural motifs, *PRND* encoding

doppel and *SPRN* encoding shadoo, are also present in other vertebrate species [63, 113, 114]. The prion protein is encoded on a single-copy gene which has been mapped to chromosome 13 in sheep, cattle, and goats, but the genomic location is unknown for cervid species. The functional length of the gene is approximately 21kb and in ruminants it consists of two introns and three exons. The introns are variable in length and may work together with the promoter regions to regulate the expression of PrP in specific tissues [115, 116]. The third exon encodes the open reading frame (ORF) of the prion protein gene, which is typically 256 codons in ruminants, and is translated and processed to a mature PrP^C protein of 210 amino acids. A significant amount of research has focused on the sequencing of this gene in a wide variety of species, to investigate the mechanisms that link PrP^C sequence strongly with susceptibility and resistance. The first and most common type of sequence variant identified are single nucleotide polymorphisms (SNP) in the DNA sequence which may be non-synonymous (producing a single amino acid change in the protein) or synonymous (silent/no change). The second and less common sequence variant is the insertion or deletion of characteristic repeat regions located in the N-terminal domain of the protein. These regions are called octapeptide repeats as they are repeating areas of 8 amino acids (PHGGGWGQ)[57].

1.4.2 PRNP genetics and prion disease

In humans 10-15% of prion disease cases are genetic familial forms of prion disease which have been associated with a variety of different causative mutations in *PRNP* including non-synonymous SNPs, octapeptide repeat insertion (>8 repeats) and premature stop codons [117].

The relationship between *PRNP* genetics and modulation of prion disease is perhaps best understood in sheep and humans, where susceptibility or resistance are controlled by *PRNP* polymorphisms at codons 136, 154 and 171 in sheep and in humans by codon 129. In sheep, representing the resulting alleles as the amino acids at these positions, for example A₁₃₆R₁₅₄Q₁₇₁ (ARQ), five commonly identified alleles (ARQ VRQ, AHQ, ARR, ARH) can be combined to

produce 15 genotypes, which confer differing susceptibility to classical scrapie [57]. These genotypes were used to create a scrapie risk-classification system, which formed the basis of the National Scrapie Plan (NSP) [118]. The NSP was a voluntary scheme for selective breeding of resistant genotypes and was implemented across the UK from 2001-2009 (with similar programmes across Europe and the USA), successfully reducing the incidence of classical scrapie. Of the naturally observed genotypes, ARR/ARR is the most resistant, though rare cases of classical scrapie have been documented in sheep of this genotype [119], and the success of the NSP has been challenged by the susceptibility of these animals to BSE [120] and atypical scrapie [121].

The polymorphism identified in human *PRNP* at codon 129 encoding either methionine or valine has also been well studied due to its modulation of susceptibility to CJD and kuru [117]. Almost all cases of primary vCJD have been homozygous for methionine (129M), with a recent published case in a heterozygous (129MV) individual [122]. An interesting relationship exists between *PRNP* heterozygosity and susceptibility to prion disease. In humans, heterozygous 129M/V individuals also have a lower frequency of sCJD than the normal population [123]. In animals, heterozygosity is often associated with prolonged incubation periods and/or reduced susceptibility [57]. One proposed mechanism for this inhibition is the incompatibility of the two different PrP^{Sc} molecules produced in a heterozygous animal, which impairs the formation of amyloid fibrils [124]. Alternatively, expression of one susceptible and one resistant allele at 50% may simply lower the availability of compatible PrP^C substrate, supported by the finding that heterozygous sheep expressing both a susceptible (VRQ) and resistant (ARR) allele only accumulated PrP^{Sc} made up of the VRQ protein [125].

The mechanisms determining the effect of an individual amino acid variation depends both on the specific substitution, and the position in the sequence of PrP. This may in turn effect the ease with which prion protein variants misfold into specific conformations, or possibly the affinity for strain-specific cofactors. Alternatively, it may be that resistance-associated proteins have a shorter half-

life *in vivo*, as generally resistance associated substitutions decrease the stability of the protein, which may increase PrP turnover and reduce time available for misfolding [126].

1.4.3 *PRNP genetics and species barriers*

Generally, prion diseases are transmitted more readily between individuals of the same species, and the reduced transmission efficiency when transmitting disease to another species is referred to as the species barrier. Species barriers are controlled by the homology of the donor and host *PRNP* sequence, strain-dependent structural compatibility of the PrP^{Sc}, route of infection and dose, and other host factors [57, 127, 128]. Key differences at certain amino acids are thought to interfere with the conversion and replication process with profound impacts upon susceptibility [129, 130]. The apparent resistance of some species such as rabbits and dogs to TSEs is mostly due to the sequence of the prion protein expressed rather than other species-specific background effects, as shown by the susceptibility of transgenic rabbits expressing ovine prion protein to scrapie [131]. In dogs, resistance to infection has been linked to the presence of aspartic or glutamic acid at codon 163 (PrP-163), which confers resistance to conformational change *in-vitro* and challenge with prion isolates *in-vivo* [132]. Conversely, bank voles are uniquely susceptible to prion diseases; the adaptation of CWD prions in bank voles produced the fastest prion strain to date with an incubation period of just 35 days, and overexpression of bank vole PrP^C in transgenic mice leads to spontaneous prion disease [96, 133–135]. Transgenic mice expressing bank vole PrP carrying the critical dog amino acid substitution (N159D, equal to canine 163Asp) had delayed propagation of both CWD and spontaneous prion isolates described above, demonstrating the critical importance of a single amino acid change [91].

Accumulating evidence shows that specific amino acid residue differences between species in the β 2- α 2 loop region of the prion protein are closely related to changes in protein structure and the species barrier [136–141]. The β 2- α 2 loop is a structure connecting the second anti-parallel β -sheet (β 2) and the

second α -helix ($\alpha 2$) in the C-terminal globular domain of PrP (Figure 1-1). In cervids, this loop region encompasses residues 168-178, is well-defined and rigid compared to other species due to amino acid substitutions at codons S173N/N177T (170 and 174 mouse numbering) [142]. Transgenic mouse models expressing either rigid (170N, 174T) or disordered loop (170S, 174N) variants showed that the similarity between loop region structure is a key determinant of cross-species transmission [137]. Species with a 'flexible loop' such as mice and humans have been hypothesised to be more resistant to prion disease from 'rigid loop' species such as cervids [136, 142]. Transgenic mice engineered to express human PrP with the elk $\beta 2$ - $\alpha 2$ loop to produce human/elk chimeric PrP were highly susceptible to CWD prions, whereas mice expressing human PrP were resistant. Interestingly, mice expressing human/elk PrP were more resistant to human CJD prions [140].

1.5 Prion strain characteristics

Experimentally, the species barrier manifests as either lower attack rates (lower percentage of animals becoming infected) or as extended incubation periods in the new host. Repeated passage of an isolate in a new host leads to 'adaptation' with higher transmission efficiencies and reduced incubation periods. After adaptation, characteristic heritable phenotypes emerge which are defined by length of incubation period, clinical signs, distribution of neuropathologic lesions (vacuolation profile) and disease associated PrP deposition (PrP^{Sc}), tissue tropism, and PrP^{Sc} biochemical differences [130, 143–145]. These characteristics form the basis of 'strain typing', whereby the passage of an isolate into a new host (usually in small rodent models such as inbred laboratory mouse lines) is the "gold standard" for the differentiation of prion strains [143, 146].

The first indication of the existence of prion strains was observed in goats in 1961, where infection and iterative passages of scrapie resulted in two different clinical phenotypes described as "scratching" and "drowsy", which the authors concluded were due to different strains [147]. Generally, the passage of two

different isolates in the same model resulting in different phenotypes leads to the conclusion that the isolates contained different prion strains, or conversely, if there are no differences it is likely the isolates contained the same strain. However, the presence of several prion strains in a single isolate has been observed [148–150]. The presence of mixed strains in isolates, as well as the mutable properties of the PrP^{Sc} agent when passaged in a new host, can make it difficult to determine the diversity of prion strains in the original inocula [145]. Historically panels of inbred laboratory mice have been used to characterise prion strains [143], however the species barrier to transmission of some TSE isolates has driven the use of alternatives, most notably bank voles and transgenic mice expressing PrP from the relevant host species [151].

The observation of prion strains is one of the most difficult and contentious phenomena in prion biology and has been used as one of the major counterarguments to the prion hypothesis, as it is difficult to explain how a protein alone could encipher strain-specific properties. The prevailing theory is that strain properties are encoded in the specific misfolded conformation of the protein, which may be impacted by host *PRNP*, as well as the presence or absence of specific co-factors for conversion [41, 144, 152–157]. The relative levels and combinations of these co-factors in different areas of the brain may play a key role in determining strain-specific tropism [157, 158]. The conformational differences between prion strains may be distinguished using various biochemical analyses, the most common of which is the electrophoretic mobility of PrP^{Sc} in polyacrylamide gels after PK digestion (detected by western blotting). The length of the PK resistant fragment, and the proportions of the PrP^{Sc} glycoforms may both differ between prion strains. Other biochemical techniques reveal differences between strains in the relative resistance to proteolytic degradation or resistance to chaotropic agents such as guanidine hydrochloride. [159] However, some prion strains, such as two strains of CWD in North America [160], are indistinguishable using PrP^{Sc} biochemical properties. The adaptation of prion strains when transferred into new host-environments (different species, *PRNP* genotype or *in-vitro* cell models) is not

fully understood. It is possible that prions exist as a 'cloud' of conformational variants in a natural host, and only the variant best suited to the new environment is selected and is propagated to form the expression of the observed phenotype [161, 162]. Alternatively in the 'deformed templating' model, it has been suggested that the passage of prions in a novel PrP^C environment drives the diversification of prion conformers in an adaptive response, which may result in the emergence of new prion strains [155, 163, 164].

1.6 Chronic wasting disease

1.6.1 *Origins and introduction*

Between 1967 and 1979 a wasting syndrome in captive mule deer (*Odocoileus hemionus hemionus*) and a single black-tailed deer (*O. hemionus columbianus*) was observed in the Rocky Mountain Laboratories in Colorado, USA, and attributed to nutritional deficiencies or captivity stress. It was not until 1978 that retrospective histopathological examination revealed characteristic spongiform change, similar to scrapie in sheep, and chronic wasting disease was recognised as a spongiform encephalopathy [165]. CWD is also similar to scrapie in the early and widespread accumulation of PrP^{Sc} in lymphoid tissues, which probably contributes to the efficiency of horizontal transmission characteristic of these two prion diseases. CWD is the only prion disease present in wild animal populations, and its concerning epidemiology includes spread throughout North America, outbreaks in Korea associated with imports of infected elk from USA, and the recent emergence in Scandinavia. Wild and farmed cervid species naturally affected with CWD include white-tailed deer (*O. virginianus*), mule deer, elk (*Cervus canadensis*), moose (*Alces alces sp*), reindeer (*Rangifer tarandus*) and red deer (*C. elaphus*). The origins of CWD in North America are unknown, and potentially include cross-species transmission of scrapie into deer, a spontaneous disease, or an unidentified source of infection. The scrapie theory has some support from the similarities in pathology and western blot profile between the two diseases [166], experimental transmission

of scrapie to elk [167, 168] and white-tailed deer [169] and *in-vitro* conversion assays [76]. However characterisation of scrapie and CWD isolates in rodent models show no common strains ([143], Diack, pers. comm.), and it is difficult to draw conclusions from experimental transmissions undertaken in cervids with small numbers of animals and not using natural transmission routes (i.e. intracerebral inoculation). The spontaneous occurrence of prion disease has been suggested to be the origin of both sporadic human and some atypical ruminant prion diseases [19, 170–172]. Support for the potential for cervids to develop sporadic CWD has been provided by the relative ease of producing *de-novo* prions using cervid PrP from transgenic mouse brain homogenates in unseeded PMCA experiments, which generated a CWD strain with unique features when inoculated into mice [173]. Additionally, the introduction of the cervid sequence in the β -2- α 2 loop region of the prion protein (see section 1.6.5.2) in transgenic mice induced a spontaneous prion disease [136]. Overall, the origins of CWD remain unclear for both North America and Norway.

1.6.2 Distribution, transmission, and control

The initial identification of CWD in wildlife facilities in Colorado was soon followed by further reports of the disease in mule and black tailed deer in Wyoming wildlife facilities, and in captive Rocky Mountain Elk (*Cervus elaphus nelsoni*). Identification of CWD in free-ranging individuals of these species in areas of Wyoming and Colorado followed, leading to these states being considered as an endemic region [174, 175]. Surveillance and modelling of CWD cases in this endemic area has suggested that disease may have been present in free-ranging animals since at least the early 1960's [176]. In subsequent years, CWD continued to expand in both host range and distribution, with cases in free-ranging white-tailed deer in Nebraska and South Dakota in 2001 [177] and Wisconsin in 2002 [178], and in wild Shira's moose in Colorado in 2005/6 [179]. Within North America, the spread of CWD is characterised by pockets of geographically distant foci, attributed to human translocation of animals, and slower spreading of disease around these areas due to natural movement and

migration of deer. The movement of animals from the US to Canada was retrospectively linked to cases of CWD in Toronto zoo between 1973 and 2003 [180]. Similarly the importation of preclinically infected elk from Canada in 1997 has been linked to outbreaks of CWD in elk in the Republic of Korea [181, 182], followed by additional cases in red deer, sika (*C. nippon*) and red-sika hybrids in 2010 [183]. At the time of writing, CWD has been identified in captive and free-ranging cervids in 26 US states and three Canadian provinces. Control of CWD in North America has posed significant challenges due to the i) efficient transmission of CWD, ii) environmental persistence, iii) human movement of cervid species, iv) historical lack of diagnostic tests for preclinical animals, v) and lack of effective control measures. Control efforts to date have been largely unsuccessful and are reviewed in [184]. The highly efficient transmission of CWD in cervid populations is unmatched by other prion diseases and is demonstrated by the high incidence of disease in some outbreaks in captive populations. The first reports of CWD in captive mule deer showed that 53 of 67 mule deer maintained in captivity for more than 2 years became positive [165]. Outbreaks on farms show similarly high prevalence, with 79% of deer affected on a Wisconsin white-tailed deer farm [185] and 59% on an elk farm in South Dakota [186]. Wild animals have lower rates, in Colorado prevalence was estimated at 5% in mule deer, 2% in white-tailed deer and 1% in elk by hunter harvested surveillance carried out 1996-1999 [176], however others report higher prevalence in Colorado which may be linked to a drop in deer abundance by 45% during 1988-2006 [176, 177]. High prevalence of CWD in a population of white-tailed deer in resulted in a 10.4% annual decline in numbers, with CWD positive deer 4.5 times more likely to die than CWD negative deer annually [187].

Using conventional as well as highly sensitive amplification methods, CWD prions have been demonstrated in a wide variety of organs and biological fluids; furthermore, infectivity has been demonstrated in saliva, faeces, urine and blood [160, 188, 197–203, 189–196]. Transmission of CWD may occur through direct contact, aerosols, maternal transmission or indirectly via persistence of

prions in the environment [204–208]. The threshold for minimum infectious dose required for CWD infection has been suggested to be between 100 to 300ng of CWD positive brain or saliva equivalent [203]. Lateral transmission of CWD prions through ingestion of contaminated forage or water is the most likely natural route. Environmental contamination may also occur through carcasses or carcase remnants left by hunters. For example, the housing of naïve mule deer in paddocks containing either positive deer 26 month prior, or a 20-months decomposed CWD-infected carcase resulted in CWD transmission [209]. Environmental persistence of both scrapie and CWD have been suggested historically by the re-emergence of disease in facilities that had attempted to eradicate disease through careful decontamination [210, 211]. The resistance of prions to degradation, their binding to soil particles (particularly montmorillonite clay) and even uptake by plants [212], all contribute to this exceptional environmental persistence. Prions bound to soil remain infectious by oral consumption [204, 213], as do contaminated plants [212]. The reservoir of infection maintained in the environment is certainly important in sustaining epidemics, and infectivity may persist in soil for an indefinite amount of time [209, 214–216].

1.6.3 Diagnosis of CWD

1.6.3.1 Clinical signs

Clinical signs of CWD are rarely seen in wild animals, however, when present they may be appreciated even by untrained observers. Most descriptions of clinical disease in the literature are based on experimental infection of captive elk and deer in North America. The youngest animal diagnosed with natural CWD and experimental oral infection suggest a minimum incubation period of 15-16 months, with an average incubation of 20-25 months in experimentally infected mule deer [217]. The maximum incubation period of wild deer is unknown; however, cases generally occur in animals aged 3 to 7 years. The duration of clinical signs varies from a few days up to a year, with the average surviving between a few weeks and 4 months in captivity, and likely shorter in

the wild. Subtle behavioural changes may be the first detected clinical signs and include increased or decreased interaction with humans and other animals, repetitive behaviours, and depression. Progressive loss of condition occurs due to reduced (but not absent) appetite. At later disease stages there is cachexia (marked wasting), which may be in combination with polydipsia and polyuria, ptyalism (excessive salivation), bruxism (teeth grinding), difficulty swallowing, incoordination and ataxia, wide based stance and head tremors [177, 217].

1.6.3.2 Differential diagnoses, gross and clinical pathology

Differential diagnoses for CWD include any diseases causing neurological clinical signs or emaciation, and include encephalitis with acute haemorrhagic disease, meningeal worm (*Parelaphostrongylus tenuis*) or locoweed intoxication. Aspiration pneumonia may be seen secondary to CWD due to difficulty swallowing and may lead to misdiagnosis in captive herds. Changes identified upon post-mortem examination of affected animals are likewise non-specific; deer may be in poor or good nutritional state depending on the length of clinical phase, and if associated with aspiration pneumonia, there may be a fibrinous pleuritis. Haematology and biochemistry are often unremarkable with the exception of low urine specific gravity due to polydipsia [177]. Clinical signs and post-mortem examination therefore cannot be used to provide definitive diagnosis of CWD.

1.6.3.3 Diagnostic tests

The diagnosis of CWD relies on the same tests described for TSEs in general, as described in section (1.3.4). The histologic lesions of CWD are similar to those of other ruminant and human TSEs, with characteristic grey matter vacuolation in the CNS, accumulation of PrP^{Sc} and gliosis [218, 219]. The earliest lesions are detectable in the dorsal motor nucleus of the vagus nerve (DMNV), followed by adjacent nuclei in the brain stem, thalamus, hypothalamus and olfactory cortex. Lesions may become widespread throughout brain regions, with relative sparing of the cerebral cortex, hippocampus and basal ganglia. Amyloid plaques

are particularly characteristic of CWD [220–222], though are also present in a number of other TSEs including kuru [223], sCJD [224] vCJD [10], and murine scrapie [225], but are less common in BSE and scrapie. Amyloid plaques may be visualised on haematoxylin and eosin stained sections (H&E) as slightly pale eosinophilic rounded fibrillar structures in the neuropil, and may be surrounded by vacuoles (florid plaques). Plaques may be visualised with silver stains, Congo-red, thioflavin-S, or by immunohistochemistry for the prion protein. The distribution of PrP^{Sc} in the brain is typically similar to vacuolation but may occur in areas with no spongiform change. Deposition is widespread, predominantly in the grey matter, and types of deposition include perineuronal and perivascular, extracellular plaque-like and granular deposits, subependymal and subpial deposition [177, 218, 219]. PrP^{Sc} deposition in lymphoid tissues is often but not always a feature of CWD and occurs prior to detection in the CNS [102, 217, 226]. Sampling of easily accessible rectoanal mucosal-associated lymphoid tissues (RAMALT) is useful for the detection of pre-clinical disease in live captive animals [227]. Rapid tests including TeSeE (Biorad) and HerdCheck (IDEXX) (described in section 1.3.4) initially developed for BSE or scrapie surveillance are also useful in the detection of CWD, and are reviewed with performance data in an EFSA report published in 2018 [228]. Confirmatory tests for any positive results are undertaken using immunohistochemistry and/or western blotting. *In-vitro* amplification of CWD has been achieved using PMCA and RT-QuIC (1.3.5). PMCA experiments efficiently amplified CWD prions using brain homogenate from deer PrP^C expressing transgenic mice (TgCerPrP1536) [178]. The PMCA products were demonstrated to maintain infectivity *in-vivo* using the same transgenic mice with unaltered strain characteristics [229, 230]. PMCA and RT-QuIC have been developed for use as diagnostic assays in pre-clinical animals, for example the detection of CWD in nasal brushings, RAMALT tissue sampling or blood [201, 231, 232]. However, these tests are currently under development and not yet routinely used in surveillance settings [228].

1.6.4 *Experimental transmissions and species barriers*

1.6.4.1 *Cervids*

The ease of transmission between cervid species has been demonstrated by natural cases of CWD in a variety of cervids in North America. A number of experimental transmission experiments have also been performed in small groups of cervids, including those known to be susceptible in North America such as mule deer [174, 226, 233], white-tailed deer [189, 234, 235] and elk [236–238]. Three captive Shira's moose showed spongiform change and PrP^{Sc} deposition after oral inoculation with CWD, however died of unrelated causes [239]. The susceptibility of other cervid species has likewise been demonstrated through experimental CWD transmission to species such as muntjac deer (*Muntiacus reevesi*) [206], European red deer [240], and reindeer [241, 242]. In reindeer, CWD from white-tailed deer, but not elk, was transmitted orally [241], whilst CWD inocula from white-tailed deer, elk and mule deer were all successfully transmitted by intracerebral injection, and horizontal transmission was achieved by direct contact or via environmental contamination [242]. The experimental infection of fallow deer has provided interesting results. Fallow deer inoculated intracerebrally with white-tailed deer and elk CWD prions showed no evidence of infection at 7.6 months post infection (MPI)(n=3), limited accumulation of PrP^{Sc} in the absence of vacuolation between 24 and 37 MPI (n=5), and both PrP^{Sc} and spongiform encephalopathy between 51 and 60 MPI (n=5) [243, 244]. Whilst these reports show that intracerebrally inoculated fallow deer can support amplification of CWD prions and develop a spongiform encephalopathy, the prolonged incubation times and relatively mild clinical signs suggest resistance in this species. This is further supported by the finding that fallow deer do not develop CWD when exposed to a contaminated environment by co-housing with infected mule deer over a period of 6 years [245].

1.6.4.2 Other animal hosts

Whether CWD transmission extends beyond the cervid family is currently unclear. Transmission to a wide variety of species including mice, voles, hamsters, racoons, ferrets, mink, cats, pigs and squirrel monkeys has been demonstrated experimentally [135, 246, 255–257, 247–254]. However, as experimental transmission often employs unnatural routes of infection such as intracerebral inoculation, whether CWD transmission to these species would occur naturally is less clear. The role of potential spillover wildlife species in the ecology of CWD is an area of interest [250, 258]. Mountain lions are known to selectively prey upon CWD infected deer [259], however both genetic studies [64] and exposure of three captive mountain lions to infected carcass material for >15 years has provided no evidence of susceptibility to CWD (Miller, pers. comm). Whilst sympatric wild animals may not be acting as reservoirs of CWD infection, their movements may play a role in the spread of CWD, for example CWD prions in the faeces of scavenging species such as crows remain infectious [260]. Much work has focused on the potential for transmission to livestock species such as sheep, cows and pigs, but experimental transmissions and transgenic mouse experiments suggest a strong barrier to infection [252, 261–268].

1.6.4.3 Humans

Given the lessons learned with the UK BSE epidemic and emergence of vCJD, there has been considerable concern surrounding the zoonotic potential of CWD. In North America, as CWD prevalence increases year-on-year so too does the likelihood of human exposure. CWD prions are present in skeletal muscle [188] and fat [269], and CWD cases are over-represented among hunter-harvested animals in some endemic parts of the United States [187]. Despite the likely exposure of humans to CWD, reports of unusual CJD cases, or CJD cases in active hunters, have been invariably linked to either sporadic or genetic CJD [270, 271] and epidemiological surveys have reported no links between CWD and human cases of prion disease [272–274]. A retrospective study did not find

an increased relative risk of CJD in an CWD endemic area (Colorado) compared to non-endemic areas [273]. In experimental transmissions to non-human primates (squirrel monkeys and cynomolgus macaques), squirrel monkeys were susceptible with 80% attack rates for intracranial inoculation and 15% attack rates after oral transmission [247, 249]. Cynomolgus macaques (regarded as evolutionarily more closely related to humans), and transgenic mouse models expressing human PrP, have both failed to demonstrate disease following infection with CWD by any attempted transmission route [140, 268, 275–277]. However, a second study using cynomolgus macaques undertook CWD challenge through a variety of routes including oral inoculation with CWD infected muscle tissue. This study is unpublished, however preliminary data has reported a neurodegenerative disorder in five of 18 challenged animals in association with faint PrP^{Sc} accumulation and seeding activity [278], renewing concern for the zoonotic potential of CWD.

In-vitro models have also been used to examine the robustness of the species barrier between humans and animal prion disease [279]. Conversion of the human prion protein in PMCA assays have demonstrated using CWD seeds from elk, WTD and reindeer, and was affected by human *PRNP* genotype at codon 129 [280–282]. Initial RT-QuIC studies showed that the conversion of human rec-PrP was more efficient for CWD (WTD) than cBSE seeds, though less so than sCJD [283]. Further RT-QuIC studies also showed the efficient conversion of human rec-PrP using WTD, elk, mule and reindeer seeds [284]. However, despite conversion of human prion protein by CWD in *in-vitro*, these assays use a reductionist approach that isolates the effect of PrP sequence and structure and may not capture other important host factors necessary for zoonotic transmission.

Overall, there remains a high level of uncertainty surrounding the potential for zoonotic transmission of CWD. The majority of the evidence including epidemiological surveys in North America and lack of transmission in animal bioassays, supports that the risk is low. However, considering the adaptation of CWD to new PRNP genotypes and the unknown impact of CWD strain variation

upon cross-species transmission, the prolonged incubation periods and difficulty of diagnosing prion disease in humans, the potential for zoonotic transmission of CWD should be considered a risk until proven otherwise [276].

1.6.5 Cervid PrP genetics

1.6.5.1 Effects of cervid prion protein gene (PRNP) variation on susceptibility

The coding sequence of the open reading frame of the gene encoding PrP, referred to as *PRNP*, is well conserved in cervids. However, a number of *PRNP* polymorphisms have been identified that are associated with lower rates or slower progression of CWD in North American cervid species. The earliest identified was a polymorphism at codon 132 in Rocky Mountain elk which encodes either methionine (M) or leucine (L) [285]. This codon was of particular interest as it corresponds to codon 129 in humans which encodes either methionine (M) or valine (V), and significantly affects prion disease phenotype and susceptibility [117, 123, 286]. Likewise in elk, animals homozygous for methionine at codon 132 (132MM) were over-represented in both free-ranging and farmed populations with CWD, compared to unaffected populations, suggesting that 132L may have a protective effect [287]. This has been supported by surveys in captive and wild elk [232, 288], however these results were contradicted in one study found that the genotypes of free-ranging elk in Colorado positive for CWD were present in proportion to their frequency in the population [289]. Nonetheless, experimental oral transmissions of CWD in elk supported reduced susceptibility in elk expressing L132 [236–238], showing that 132LL homozygous animals had incubation periods 1.5 times longer than 132ML elk, and 3 times longer than 132MM elk [236, 237]. The variation in incubation period between 132MM, ML and LL elk was associated with differences in neuropathology, western blot phenotype, PrP^{Sc} fibril stability and amyloid formation rate. 132LL elk had accumulated relatively less PrP^{Sc}, showed higher fibril stability and PrP^{Sc} amplified more readily in 132L compared to 132M recombinant substrate by RT-QuIC [238].

A number of genetic surveys have examined *PRNP* polymorphisms in wild and farmed white-tailed deer and mule deer in North America, identifying polymorphisms such as G96S, Q95H, A116G, Q226K/R and Q230L in white-tailed deer, and D20G and S225F in mule deer [290]. Early studies were complicated by the identification of a processed but unexpressed *PRNP ψ* pseudogene, which was not correlated with CWD status [291–294]. Analysis of 133 white-tailed deer in Nebraska revealed three polymorphisms, at codons 95 (Q/H), 96 (G/S) and 116 (A/G)[293]. Free-ranging Wisconsin white-tailed deer also encoded these polymorphisms, as well as an additional rare SNP at codon 226 (Q/K). Comparison the frequencies of the predominant alleles identified in white-tailed deer with and without CWD suggested that 96S and 95H alleles were linked to reduced susceptibility. Lower levels of PrP^{Sc} deposition were identified in the brains in heterozygous animals (96G/96S), however the 96S allele did not provide full protection, as demonstrated by a CWD-positive 96SS homozygote animal [295]. Additional genetic surveys in CWD-affected areas provided further evidence that the 96S allele is consistently associated with lower CWD prevalence compared to the 96G allele [185, 296]. The 96S allele may provide a significant fitness advantage in a CWD endemic area, with 96G homozygous deer having a four times greater risk of CWD infection [296]. Whilst the 95H and 116G alleles occur less frequently in white-tailed deer populations, similar genetic surveys have also suggested relative protection in deer expressing these alleles [293, 295, 297–299]. Experimental challenge further demonstrated the effects of polymorphisms on CWD progression; twelve WTD of varying genotypes (95Q/96G, 95Q/96S, 95H/96G and 95H/96S) were orally challenged with pooled brain homogenate from natural CWD cases in WTD expressing the wild-type allele (95Q/96G)[234]. All deer displayed clinical signs of CWD, but varied in survival period depending on genotype with an average survival period of 693 days post inoculation (dpi) for 95Q/96G deer, increasing to 956, 1507 and 1695 dpi for 96Q/96S, 95H/96G and 95H/96S respectively [234]. Not only did the 95H allele appear to significantly impact the survival period of infected deer, it was also associated with reduced accumulation of PrP^{Sc} in peripheral organs [235]. RT-QuIC analysis of the

progression of CWD in WTD by analysis of peripheral lymphoid tissue (RAMALT) reinforced the findings from earlier experimental transmissions, with delayed IHC detection of PrP^{Sc} in lymphoid tissues of deer indicating a slower progression in deer with 96GS, 96SS and 95H genotypes [300]. *In-vitro* analyses such as RT-QuIC are also useful for estimating the effects of *PRNP* polymorphisms, by analysis of amplification rates and amyloid extension efficiency [301, 302]. Aligning with *in-vivo* susceptibilities, RT-QuIC amplification rates were lower for resistance associated WTD alleles (95H, 96S, 116A and 226K) [301].

Mule deer in CWD-endemic areas of Colorado and Wyoming expressing homozygous wild-type 225S *PRNP* developed CWD at a rate 30 times higher than in animals heterozygous for 225S/F. Animals expressing 225SF or 225FF accounted for 9.3% of the CWD-negative deer, but only 0.3% of CWD-positive deer, together suggesting that the 225F allele is a significant barrier to infection [303]. Experimental transmissions corroborated this finding, as oral inoculation of mule deer showed markedly delayed disease progression in 225SF heterozygotes [226], and environmental challenge of 225SS and 225FF deer produced differences in clinical presentation and atypical pathological features in 225FF deer [233]. In transgenic mice expressing the F225 allele, SSBP1 incubation periods were prolonged threefold compared to mice expressing S225, and CWD transmission resulted in incomplete attack rates and variable incubation periods. When F225-expressing brain homogenate was used as substrate for PMCA, the amplification efficiency of CWD was reduced compared to S225 substrate [304]. RT-QuIC amplification of CWD in mule deer 225F substrate produced one of the lowest amplification rates when compared to a range of other cervid PrP substrates [301].

Whilst initially identified in the *PRNP* pseudogene of WTD and mule deer [293, 294], the presence of asparagine (N) at codon 138 has since been reported in the functional *PRNP* gene of fallow deer and reindeer. All sequenced fallow deer to date are homozygous for asparagine (N) at codon 138 and were resistant to chronic wasting disease when exposed via a contaminated environment [245,

305, 306]. Fallow deer differ from mule and WTD only at this position, suggesting 138N may confer relative resistance to CWD. Reindeer are polymorphic at codon 138, expressing either serine (S) or asparagine (N). In North America, the 138N allele is present in reindeer populations [242, 307, 308]; the frequency of this allele varies between 0.2-6.4 depending on subspecies and population [309]. One population of woodland caribou (Chinchaga) had a significantly higher frequency of the 138N allele, with 36.7% of animals sampled homozygous for the 138N allele [308]. This polymorphism has not been detected in surveyed Norwegian reindeer populations [310]. Reindeer with 138SS, NS and NN genotypes were susceptible to CWD following intracerebral inoculation with CWD from a variety of sources, and transmitted infection to naïve reindeer through direct and indirect exposure [242]. However, animals with the 138NS genotype had significantly lower levels of lymphoid involvement [242], and were resistant to oral infection with CWD from WTD and elk [241]. Additionally, gene-targeted transgenic mice expressing the 138N-cervid PrP have been reported resistant to intracerebral and intraperitoneal inoculation with a variety of CWD isolates, whilst mice expressing 138S were susceptible [311].

1.6.5.2 Cervid PRNP variation and protein structure

The mechanisms controlling the effects of specific variations in the amino acid sequence of the prion protein on disease susceptibility are not fully resolved. Whilst studies investigating the structural effects of various cervid *PRNP* polymorphisms are limited, *in-silico* molecular dynamics and high-resolution structural studies using nuclear magnetic resonance (NMR) have provided some evidence of the structural effects of cervid polymorphisms. Attention has focused on the $\beta 2$ - $\alpha 2$ loop region between the second β -sheet ($\beta 2$) and the second α -helix ($\alpha 2$), which is extremely well defined and rigid in cervids compared to other species [142, 312]. The rigidity of this loop region is due to amino acid substitutions at codons 170 (S to N) and 174 (N to T) in cervid species; the introduction of these substitutions and overexpression of the 'rigid

loop' in transgenic mice results in the de novo (spontaneous) generation of prion disease [136]. The loop region has also been ascribed importance in the efficient horizontal transmission of CWD [142] and in transmission barriers, including zoonotic transmission of CWD to humans (section 1.4.3). The β 2- α 2 loop region forms a discontinuous epitope with the third alpha helix (α -helix 3)[312], a region of the PrP protein containing codons 225 and 226. Molecular dynamics analyses show that substitutions S225F and E226Q alter the orientation of residues in the β 2- α 2 loop (D170) and in the α -helix 3 (Y228), creating structural changes that allow the formation of hydrogen bonds between the side chains of these residues and reduced plasticity/increased rigidity of the β 2- α 2 loop/ α -helix 3 epitope [304]. The increased stability of this epitope may therefore contribute to the importance of codon 226 in the manifestation of prion strains (1.6.6) and the reduced susceptibility of mule deer expressing 225F.

1.6.5.3 *PRNP polymorphisms and disease management*

It is tempting to speculate whether cervid *PRNP* polymorphisms could be used in the management of CWD, following on from the success of the National Scrapie Program in sheep and goats (see section 1.4.2). However, to date there is no evidence of a *PRNP* polymorphism in cervids that is totally protective. Additional considerations for the use of selective breeding for *PRNP* resistance alleles in the control of CWD include the feasibility in wild populations, effects on overall fitness in the animal, differences in prion shedding, and the potential for the selection or generation of novel prion strains (1.6.6). Additionally, although resistance alleles may provide an advantage in CWD-endemic areas, whether the expression of these alleles confer an evolutionary fitness disadvantage compared to wild-type is largely unknown [296]. A limited but useful study in captive elk in Colorado found that the 132L allele was associated with reduced susceptibility, and did not appear to affect overall fitness as determined by pregnancy rates, body condition score and calf survival [313].

The detection of prion shedding in various bodily fluids is sustained through the disease course and occurs as early as 3 months after CWD exposure [198]. The prolonged survival of cervids expressing *PRNP* alleles associated with reduced susceptibility may therefore contribute to prolonged shedding, leading to increased environmental burden of prions. Experimental oral inoculation of mule deer demonstrated similar levels of accumulation and distribution of PrP^{Sc} in the CNS of 225SS and 225SF expressing animals. However, whilst lymphoid deposition occurred at similar time points in the course of infection, deposition in the CNS was markedly delayed in 225SF mule deer, prolonging survival and potential shedding period [226]. Additional studies using amplification assays have produced conflicting evidence, in some cases showing decreased shedding in resistant genotypes such as the 96GS genotype in white-tailed deer [200, 314], and in others finding no effect of genotype on shedding [197, 199]. It is worth considering that these studies were performed using various experimental techniques and on small sample sizes, and more research in this area is required.

1.6.6 CWD strains

The existence of multiple strains of CWD has been suggested during the transmission of CWD into a variety of cervid and non-cervid *PRNP* expressing models [138, 149, 320–323, 234, 268, 302, 315–319]. Early studies were limited, but supported the feasibility of using transgenic mice expressing cervid prion protein for CWD strain typing [160, 229, 315, 316]. A collection of CWD isolates from wild and captive cervid species across varied geographical locations in the US were assessed by bioassay in transgenic mice overexpressing deer PrP (Tg(CerPrP)1536). This study provided evidence of two naturally occurring prion strains, CWD1 and CWD2, which have distinct clinical and neuropathological phenotypes [149]. Interestingly, whilst elk harboured either CWD1 or CWD2 prions, deer appeared to propagate CWD1/CWD2 strain mixtures, a finding that corroborated previous transgenic mice studies suggesting cervid brain inocula may contain mixtures of strains [268]. It is

unknown whether these two prion strains act synergistically, perhaps facilitating the transmissibility of natural CWD, or if they interfere, as has been previously reported for other strains [324–326]. The primary difference between deer and elk PrP sequence is the presence of glutamine (Q) or glutamic acid (E) at residue 226 respectively, and experimental inoculations of deer and elk show differences in disease progression and lesser lymphoid involvement in elk compared to deer [327]. Studies in transgenic mice expressing 226E and 226Q proteins inoculated with elk CWD resulted in prolonged disease onset in mice expressing deer 226Q by 20-46% and differences in neuropathology between the two mouse lines [160]. Further experiments either in overexpressing transgenic [149] or gene-targeted [320] mice indicate that amino acid differences at residue 226 determine the selection and propagation of CWD strains. The conformational stability of prions, determined by denaturation with guanidine hydrochloride, can be used to differentiate prion strains. PrP^{Sc} produced in either transgenic mice or RK13 cells expressing cervid PrP shows higher conformational stability of deer 226Q compared to elk 226E [149]. Transmission in transgenic mice expressing 226E (elk) resulted in the propagation of more stable strains than in mice expressing 226Q (deer), which were less stable and often resulted in mixed strain profiles [149]. In addition, the 225F allele has been associated with a distinct CWD strain, as demonstrated by incomplete attack rates, variable incubation periods, and differences in PrP^{Sc} distribution patterns in transgenic mice expressing 225F [304]. These effects are likely due to the conformational differences described in section 1.6.5.2.

Transmission of CWD isolates to transgenic mice expressing elk *PRNP* genotypes, showed that transgenic mice expressing the MM132 genotype (TgCerPrP) were susceptible to CWD from elk with 132MM, ML or LL genotypes. In contrast, the same isolates failed to transmit to mice expressing the elk LL132 genotype (TgCerPrP-132L) [328]. Recently, phenotypic characteristics observed upon experimental transmission in elk (1.6.5.1) were shown to be retained upon passage to transgenic mice expressing the elk (M132) prion protein [323]. Compared to mice inoculated with CWD from

132MM donors, 132LL material resulted in longer incubation periods, lower molecular weight of PrP^{Sc} on western blot, increased fibril stability and differences PrP^{Sc} deposition in the brains of tg mice. These characteristics, with the exception of fibril stability, were maintained on secondary passage, suggesting that a novel strain is propagated in 132LL elk [323]. In combination with reports of two strains of naturally occurring CWD in Elk [149], this brings the potential total of elk CWD strains to at least three [323].

In white-tailed deer, two different strains of CWD have been isolated upon passage to hamsters [322], ferrets [321] and transgenic mice expressing deer PrP [149, 318]. Passage of white tailed deer CWD to hamsters produced an unusual wasting phenotype proposed by the authors to be a prion-induced endocrinopathy [255]. The development of transgenic models expressing either 96G (tg30) or 96S (tg60) has enabled investigations into the effects of this polymorphism and CWD strain properties. Mice expressing 96G were highly susceptible to infection with incubations periods of >160dpi, displaying typical vacuolar pathology and PrP^{Sc} deposition in the brain, spleen and intestinal tissue. In contrast, mice expressing 96S showed no evidence of transmission by 600dpi [329], even when challenged with CWD inocula from a 96SS genotype deer [330]. However, in an alternative transgenic mouse model expressing deer 96S, mice were only partially resistant to CWD [304], which is in agreement with the observation of CWD-positive deer homozygous for the 96S allele [185]. The relative protection provided by the 96S and 132L alleles in transgenic mice was overcome in both models by inoculation with the experimental sheep scrapie isolate SSBP1, suggesting this resistance was at the level of strain selection [304, 328]. To further explore this, CWD inocula prepared from WTD with four different PrP genotypes (wt/wt, wt/96S, 95H/wt or 95H/96S) were transmitted to transgenic mice expressing either 96G (tg33) or 96S (tg60) alleles [318]. In tg33 mice the same strain was amplified (Wisc-1) regardless of inocula, which is consistent with the previously observed susceptibility of transgenic mice expressing 96G. Interestingly, the protective effects observed in mice expressing the 96S allele were overcome when inoculated with prions

from deer with the 95H allele (95H/wt or 96H/96S), and serial passage resulted in the propagation of a novel CWD strain (H95+). Transmission of first-passage H95+ into tg33 (wild type) mice produced two disease phenotypes consistent with the propagation of a mixture two strains. This indicates that deer expressing the H95 polymorphism are capable of propagating a mixture CWD strains, the propagation of which depends on the recipient host. As well as transmissibility in wild-type and 96S expressing transgenic mice, the H95+ strain (present in inoculum from deer expressing the 95H allele) is transmissible in wild-type (C57) mice, with clinical disease after ~575dpi (H95/S96 inoculum) or ~692dpi (H95/wt inoculum), whereas inocula from deer without the 95H allele (Wisc-1 strain[318]) or from Elk (CWD2 strain[149]) failed to transmit [319]. The reverse was observed upon transmission to hamsters, where Wisc-1 or CWD2 strains were transmissible, but hamsters were less susceptible to H95+ prions. Recent work employing a range of conformation dependent assays has demonstrated that the H95+ strain is a product of the replication of Wisc-1 CWD in hosts expressing H95 PrP^C [155]. Comparison of Wisc-1 prions with an inocula sourced from a heterozygous 116AG white-tailed deer in transgenic mice expressing wild-type white-tailed deer protein (tg(cerPrP1536+)) showed delayed disease progression, prolonged survival and distinct biochemical features in mice inoculated with the 116AG isolate. Importantly, inoculation of 96S expressing transgenic mice (tg60) mice produced survival times and biochemical differences different from the H95+ strain [311]. Additional differences in cell culture and RT-QuIC amplification characteristics supported the conclusion that deer heterozygous at residue 116 propagate a distinct CWD strain different from Wisc-1 and H95+ in white-tailed deer [302].

Differences between laboratories in the transgenic mouse models, CWD inocula and experimental transmission methods and reporting make it very difficult to accurately determine the number of CWD strains circulating in North America. However, the data available suggests that the emergence of novel CWD strains may be driven by transmission into hosts with different primary PrP^C

structures, reinforcing the importance of understanding *PRNP* genetic variability. This hypothesis is supported by a range of conformational and amplification assays demonstrating the adaptive diversification of prion conformation after passage through hosts with distinct PrP^C sequences and the competitive emergence of new prion strains [155, 331].

1.6.7 CWD in Europe

1.6.7.1 Surveillance prior to 2016

Prior to 2016, Europe was considered to be free of CWD. A survey undertaken by the European Food Standards Agency (EFSA) between 2006 and 2010 tested 13,000 brain stem samples provided from fallen or culled cervids in 21 European Union member states (MS) and Norway. An additional subset of 13 MS performed active surveillance of 600 wild or farmed animals of presumed susceptible species. No positive results were recorded, and it was concluded that there was not a high prevalence of CWD in the EU. However, the survey had several limitations including failure to meet target sample sizes by some MS, a lack of geographical representation within MS, and minimal testing in species such as reindeer, moose and roe deer. Most animals sampled were red deer, with the inclusion of white-tailed deer in Finland. Tests were performed on brain stem samples (obex) which may have excluded the detection of pre-clinical animals with PrP detectable in lymphoid tissues only. In view of these limitations, the authors noted that the “occurrence of cases of TSEs, especially in remote and presently unsampled geographic areas, may not be excluded in cervids in the EU” [332]. Testing in the following years 2011-2014 was very minimal, with a few European countries reporting low levels of testing and no positive results to EFSA [333]. Additional studies have been performed with the aim of detecting CWD in Germany [334], Norway [335] and Spain [336], again with negative results.

1.6.7.2 CWD cases in Europe

The first European case of CWD, and the first natural case in a reindeer was identified in Norway in March 2016. This chance discovery was due to the presence of a team of researchers from the Norwegian Institute for Nature Research placing radio-collars on free-ranging reindeer in the Nordfjella mountain region of Norway. A missing dart forced the team to land, which led to the identification of a recumbent female reindeer separated from the main herd, which died shortly after discovery. Post-mortem examination followed by histopathological analysis, rapid-testing (TeSeE ELISA Bio-Rad) and western blotting confirmed the diagnosis of CWD in this individual [17]. Increased surveillance for CWD in Norway since 2016 has involved testing of over 125,000 cervids including reindeer, moose, red deer, roe deer and fallow deer. To date, Norway has reported CWD in 20 reindeer, eight moose and a single red deer [337, 338]. To determine the prevalence and geographical spread of CWD in Europe, a three-year surveillance program starting in 2018 was adopted for countries with reindeer and/or moose populations including Finland, Sweden, Latvia, Lithuania, Estonia and Poland. The aim was for each of these countries to survey 3,000 wild or semi-domesticated cervids during this time, however progress has been slow due to logistical constraints and the efficacy of this surveillance is not yet clear. A total of 5,110 animals from these countries were tested in 2018. This surveillance has led to the detection of further CWD cases in Moose; in Finland, two cases were identified in February 2018 and October 2020, and in Sweden four cases in March, May and September 2019, and September 2020 [339, 340]. No cases have been identified in farmed or semi-domesticated cervids, or in roe, white-tailed deer or fallow deer, however the numbers of animals tested from these species are lower than for reindeer, moose and red deer. The overall prevalence of CWD in European countries remains unknown, and data are limited for Sweden and Finland. Recent studies analysing data from the Nordfjella region of Norway have estimated CWD prevalence at 1.2% (true prevalence 1.6%) of adults in the infected population of reindeer [341]. The nineteen positive reindeer identified prior to 2020 were

confined to zone 1 Nordfjella mountain region of Norway however the first identification of a positive reindeer outside this region was reported in September 2020. This reindeer was located on the Hardanger plateau in central Southern Norway, south of Nordfjella (Figure 1-3). To date, this brings the total number of cervids identified with CWD in Europe to 35 [337, 340, 342].

In Norwegian reindeer positive for CWD, the presence of PrP^{Sc} in lymphoid tissues, including animals with lymphoid deposition without CNS deposition, is consistent with the pathogenesis seen in North American cervids infected with CWD. The western-blot profile and immunohistochemical deposition patterns were similar to North American CWD, including Canadian reindeer experimentally infected with CWD through oral challenge [17, 241, 242]. These findings, as well as the clustering of cases in the Nordfjella region, are all suggestive of a CWD outbreak with horizontal transmission and environmental persistence consistent with North American or 'classical' CWD. However, it is important to consider the impact of CWD strain and differences in *PRNP* genetics, which may also impact the pathogenesis and level of infectivity in Norwegian reindeer cases.

Interestingly, analysis of the first moose cases identified in Norway showed a common neuropathological phenotype characterised predominantly by intraneuronal staining, which differs markedly from the staining patterns seen in Norwegian reindeer and North American cervids (Table 1-2). Where lymph nodes were available for testing, no PrP^{Sc} deposition was identified. Western blotting of the moose isolates produced a banding pattern that was clearly distinguished from other CWD cases and most ruminant prion diseases including previous cases in North American moose. Together, this suggests a novel type of CWD in Norwegian moose. The three animals analysed shared common features on western blot such as N-terminal cleavage (preventing detection with N-terminal antibodies) and an additional C terminal fragment (CTF13), but unexpectedly varied in the presence/absence of other fragments, which may be characteristic of different strains [343]. Moose cases in Sweden and Finland have been reported to have characteristics consistent with

Norwegian findings [344], including the absence of PrP^{Sc} deposition in lymphoid tissues [344, 345]. Less information is currently available for the red deer case, however the western blot pattern has been reported as different from all previously published CWD cases, with similarities to BSE. However whilst red deer are known to be susceptible to BSE, further analysis using sPMCA has ruled it out in this case [344]. In addition to the lack of detectable deposition in lymphoid tissues, different neuropathology and western blot profiles, the European moose and red deer CWD cases are invariably identified in aged animals (Table 1-2). This has led to speculation that these cases could represent spontaneous TSEs similar to atypical BSE or scrapie, and less contagious than classical CWD [343].

1.6.7.3 Transmission studies of European CWD

Transmission studies using isolates from Norwegian CWD cases are underway in a variety of small rodent models (summarised in [344]), to assess the strain variation in Norwegian cases and the potential for cross-species transmission. Using a traditional strain typing approach in bank voles, the fastest model for CWD transmission [135], transmission of reindeer and moose isolates has provided evidence of different strains in Norway compared to North America, and differences between reindeer and moose [346]. Primary transmission of CWD isolates were characterised by varied incubation times, with Canadian isolates producing short survival times, and longer survival times with Norwegian moose isolates. Norwegian reindeer isolates were less transmissible to bank voles with only one positive transmission (1/21) after primary transmission of a reindeer CWD isolate. Upon primary transmission both the neuropathological features and the western blot profile of Canadian isolates were similar to those of previously transmitted North American isolates [135]. The single bank vole infected with the Norwegian reindeer isolate produced a western blot profile with a higher molecular weight (MW) of the PNGase treated PrP^{Sc} than moose or Canadian CWD isolates. Norwegian moose isolates were all characterised by the presence of a C-terminal PrP^{Sc} fragment (~12-13kDa)

reminiscent of the pattern seen in the original moose brain inocula and therefore preserved upon transmission to bank voles. The patterns identified also varied depending on moose inocula, with the first moose case (M-N01) showing a higher MW than the other two (M-N02/M-N03). Secondary passage and adaptation produced shortened survival times for both Canadian and Norwegian isolates, with preservation of the western blot profiles. The neuropathological phenotype following sub-passage of the Norwegian reindeer isolate was similar to CWD isolates from Canada, and parallels the findings in the original cases [17]. A further passage in bank voles confirmed that the strain isolated from Canadian CWD isolates was the same as previously isolated from US CWD isolates [135] with a survival time of just 35 days. Despite some similarities between the Norwegian reindeer isolate both in the natural host and upon transmission to bank voles, differences identified in neuropathology and western blotting, as well as the failure to produce the characteristic 'fast' CWD strain with an incubation period of 35 days, led authors to conclude that the strain in Norwegian reindeer is different from North American CWD. Third passage of Norwegian moose isolates provided further evidence of two distinct

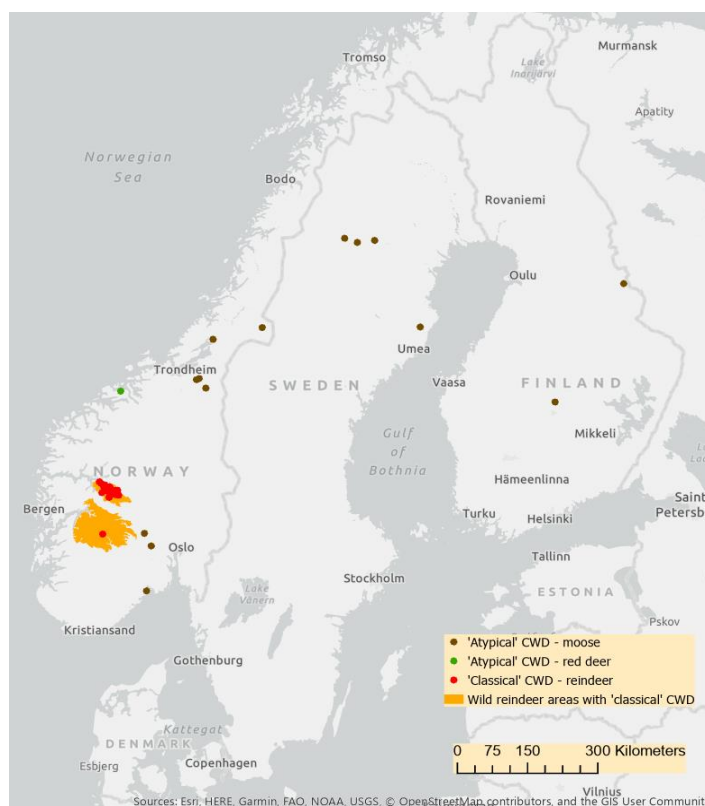


Figure 1-3: Map showing location of chronic wasting disease cases in Scandinavia.

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[<https://www.hjortevilt.no/skrantesjuka-cwd/cwd-in-norway-english/>, accessed 05/04/2021]

strains differentiated on the basis of PrP^{Sc} profile on western blot, lesion profiles and PrP^{Sc} deposition patterns. Overall, analysis of transmission in bank voles supported the isolation of four different strains, one from Canadian isolates, and three from Norwegian isolates [346].

Table 1-2: Overview of European CWD cases

Location	Species	Age	PrP ^{Sc} distribution	IHC deposition	Western blot profile	Ref
Norway	Reindeer (n=20)	Young/ Adult	Lymphoid +/- CNS	Disseminated PrP ^{Sc} , mostly in the brainstem	Indistinguish- able from North American CWD cases	[17]
	Moose (n=8)	Aged (13- 14y)	CNS only	Intraneuronal	Lower molecular weight, N-terminal truncated (atypical)	[343]
	Red deer (n=1)	Aged	CNS only	Intra and extra- neuronal, star- like	Pending, unpublished (BSE-like profile)	(345, 346)
Sweden	Moose (n=4)	Aged (16y, 16y, >10y, 14y)	CNS only	n/a*	n/a*	(338, 347)
Finland	Moose (n=2)	Aged (15y)	CNS only	n/a*	n/a*	[340]

*Preliminary results from moose cases are similar to those from Norwegian animals [344]

1.6.8 Future perspectives

The difficult and controversial decision to eradicate the Nordfjella population of reindeer was made in May 2017. This decision was based on reports and discussions from various Norwegian advisory groups, the evidence in support of intensive culling for management of CWD in the United States, and lack of other viable options [347]. It was hoped that eliminating this relatively isolated

population whilst in the early disease stages would provide a rare opportunity for CWD control [348]. In total, 2024 reindeer were culled, representing the third-largest wild reindeer herd in Norway and 7% of the total population. The two-stage eradication plan began with an extension of the normal hunting season and removal of some restrictions, however the lack of support from many local hunters meant that the second stage employed professional state marksmen to meet the target. An arbitrary fallow period of five years has been set for this area however it is uncertain if this will be effective. Prions may persist in the environment, for example the BSE-infected material can survive for at least 5 years in soil, and red deer are sympatric with reindeer in Norway and have overlapping summer range with Nordfjella [216, 349].

A recent EFSA report has divided risk factors that may contribute to the spread of CWD in Europe into 13 groups. These factors address the natural or man mediated movement or aggregation of cervids, the impact of host genetics on susceptibility, the movement of other animals contributing to spread of disease, and potential for contamination of the environment and transfer of this through fomites or movement of forage (lichens) grown on contaminated soil [344]. Unfortunately, the recent identification of a reindeer positive for the 'classical' CWD strain outside the Nordfjella region suggests that either the disease may be more widespread than surveillance suggests, or the early attempts to control disease spread have not been successful.

1.7 Thesis aims

Whilst the experience of CWD in North America provides a pessimistic view of disease spreading inexorably through European cervid populations, there are considerable uncertainties remaining. Due to inconsistent surveillance throughout Europe, it is not possible to comment on the prevalence of CWD in Europe, particularly in presently unsampled locations or cervid populations. There is strong evidence of the effects of *PRNP* polymorphism on CWD susceptibility in North American species, whilst the knowledge of *PRNP* polymorphisms in European populations is lacking. Considering the impact of *PRNP* genetics on susceptibility and potential disease transmission, as well as the mutable and evolving nature of prion strains, this information will be vital in understanding how CWD will develop in Europe.

This thesis seeks to address some of the uncertainties regarding genetic susceptibility of European deer to CWD, and strain characteristics of European CWD isolates. The hypothesis is that European CWD strains are distinct from those in North America, which will be reflected in differences in genetic susceptibility in European cervid species, and in biological and biochemical strain characteristics.

1. To investigate the extent of *PRNP* variability in the most numerous wild British cervid species, and compare this with other European and North American cervid populations to predict CWD susceptibility.
2. To predict the effects of amino acid polymorphisms unique to European cervid species on CWD susceptibility by developing *in-vitro* conversion assays.
3. To characterise the pathological phenotypes of Norwegian and Canadian CWD isolates in wild-type mice as part of a larger strain-typing effort to understand European CWD strains. Additionally, to assess if lymphoid tropism is an important feature of primary passage in wild-type mice.

2 Survey of the prion protein gene (*PRNP*) variation in British cervid species

2.1 Introduction

Until recently, CWD was thought to be a disease of North American cervid species, however since 2016 CWD has been identified in reindeer, moose and a single red deer in Scandinavia [17, 343, 345, 350]. Disease may have been present and undetected in Norway for some time, which raises the possibility that further novel instances of CWD could be uncovered in other cervid populations across Europe. The uncontrollable spread of CWD across North America since the late 1960's is a cause of great concern, and it seems inevitable that disease will spread from the population of infected Nordfjella reindeer, as evidence suggests that characteristics consistent with efficient horizontal transmission and environmental persistence are shared between North American deer and Norwegian reindeer [17, 331]. It is therefore vitally important to understand the susceptibility of European cervid populations, which differ from those predominant in North America. The prion protein gene (*PRNP*) is an important modulator of disease susceptibility in both human and animal prion diseases. In humans, variation in the amino acids at codon 129 (M/V) controls susceptibility to transmissible forms of Creutzfeldt-Jakob disease [117] and a number of variations have been strongly linked to susceptibility to prion diseases in ruminant species [57]. Perhaps most notable are the *PRNP* polymorphisms in sheep at codons 136, 154 and 171, among others, which combine to give a range of potential susceptibilities to classical scrapie, including almost complete resistance to infection in A₁₃₆R₁₅₄R₁₇₁ homozygous sheep [351, 352]. This feature was exploited in large-scale genotyping projects, such as the National Scrapie Plan in the UK and similar programmes in the European Union (EU), which have successfully reduced the incidence of classical scrapie [353, 354].

In North American cervid species, polymorphisms in *PRNP* are associated with reduced susceptibility to disease, slower progression of disease in infected animals, differences in neuropathology and lymphotropism, and the emergence of new strains of CWD. These polymorphisms are discussed in full in section

1.6.5, and include M132L in Rocky Mountain elk, Q95H, G96S, G116A, and Q226K in white-tailed deer and S225F in mule deer [290]. Determination of the effects of these polymorphisms relies on a combination of surveillance of cases (positives vs negatives of each genotype) in areas with endemic CWD, experimental transmission of disease in cervids of known genotypes, or *in-vivo* (transgenic mice) and *in-vitro* models.

A change in a single amino acid in the *PRNP* gene (as a result of a non-synonymous SNPs) can have dramatic effects on susceptibility to prion diseases and transmission barriers. The mechanisms underlying these changes remain uncertain, but are likely to involve modification of epitopes crucial for PrP^C structure, stability, and conversion to PrP^{Sc}. The prion protein consists of an unstructured flexible N-terminal domain containing a conserved stretch of octapeptide repeats and a structured C-terminal domain with three α -helices and two β -sheets [59]. Cervid *PRNP* polymorphisms in both the unstructured N-terminal, such as Q95H and G96S in white-tailed deer, as well as in the structured C-terminal, such as M132L in elk, modulate susceptibility to CWD.

Relatively little is known about the *PRNP* genetics of European cervid species. Some samples collected between 2006 and 2010 for CWD surveillance study by the European Food Standards Agency (EFSA) were also analysed for *PRNP* variation. This included a total of 132 Scottish red deer (Mainland n=82, Isle of Rhum n=50) were sequenced for the open-reading frame of the *PRNP* gene. Polymorphisms T98A and Q226E were identified in Scottish populations [355] and are also present in Italian (n=191) and Spanish (n=209) red deer populations [336, 355]. A P168S polymorphism was identified in a single red deer from the Isle of Rhum, and an additional G59S polymorphism was identified in a single Italian red deer, with additional synonymous polymorphisms at codons 15, 21, 78, 79 and 136 [355]. Since the discovery of CWD in 2016, the *PRNP* genetics of 120 Norwegian reindeer from the Nordfjella region (zone 1) have been surveyed, including the 19 reindeer that tested positive for CWD. This study found that serine at codon 225, and an octapeptide repeat deletion, were significantly associated with increased CWD risk [310].

Great Britain has six species of free-ranging deer. Red (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) are native species, whilst fallow deer (*Dama dama*) were introduced during the 11th century and are considered naturalised [356]. Muntjac (*Muntiacus reevesii*), sika (*Cervus nippon*) and Chinese water deer (*Hydropotes inermis*) are non-native species introduced just over 100 years ago [357]. Roe deer are the most widely distributed, with populations of these deer present in almost all areas of mainland Britain. Red deer, fallow deer and sika deer have populations distributed across the UK, whilst Chinese water deer and muntjac are mainly located in southern Britain (Figure 2-1). In areas of Scotland and the English Lake district populations of red and sika deer have overlapped, leading to hybridisation of these species [358]. The national deer population has previously been estimated to be at 2 million deer [359], a number which is continuing to increase [357]. However, these national estimates are uncertain due to the difficulties inherent in measuring wild deer populations. The popularity of venison has increased over recent years, as has the farming of red deer in the UK, with 37,000 farmed deer reported for 2020 [360, 361]. The UK is also home to over 270 deer parks and zoos with a wide variety of exotic deer species [362]. Whilst park and farmed deer are a small proportion of the total UK deer population, they are of high economic and aesthetic value, and importantly, may have a higher probability of exposure to external sources of CWD than wild deer given their restricted habitat range and increased interaction with tourists and returning residents [363]. Captive farmed and park deer are also more likely to be diagnosed with the subtle clinical behavioural changes noted at the early stages of CWD and are more likely to be tested and diagnosed with CWD. Reindeer are kept at a limited number of sites across the UK, including a semi-wild herd of reindeer in the Cairngorm national park, Scotland, due to the re-introduction of reindeer in the 1950's. In other areas of the UK reindeer are not well suited to the UK climate and are not well documented, though consignments of reindeer enter (usually for seasonal Christmas work). Since 2013 there have been 27 consignments of 595 reindeer from Nordic countries including 2 from Norway in 2013 and 2015, totalling 28

reindeer [363]. However, since 2016 live cervid exportation has been banned in Norway.

The introduction or identification of chronic wasting disease in the UK would have devastating consequences for both farming and stalking industries as well as wild deer populations due to the challenges of disease eradication. Control and management practices for CWD would centre around our current knowledge of natural and farmed deer population numbers, locations and movements, and importantly, the susceptibility of British species to CWD. This chapter includes a published manuscript of a large-scale survey undertaken to assess the genetic variability in the open-reading frame of the prion protein gene (*PRNP*) of wild/free-ranging deer in Britain. This survey utilised DNA samples from pre-existing archives, the collection of samples via collaboration with members of the British Deer Society, and two European red-deer surveys from the Czech Republic and Norway. Additional data presented in this chapter includes analysis of a number of exotic deer species present in parks and zoos across the UK.

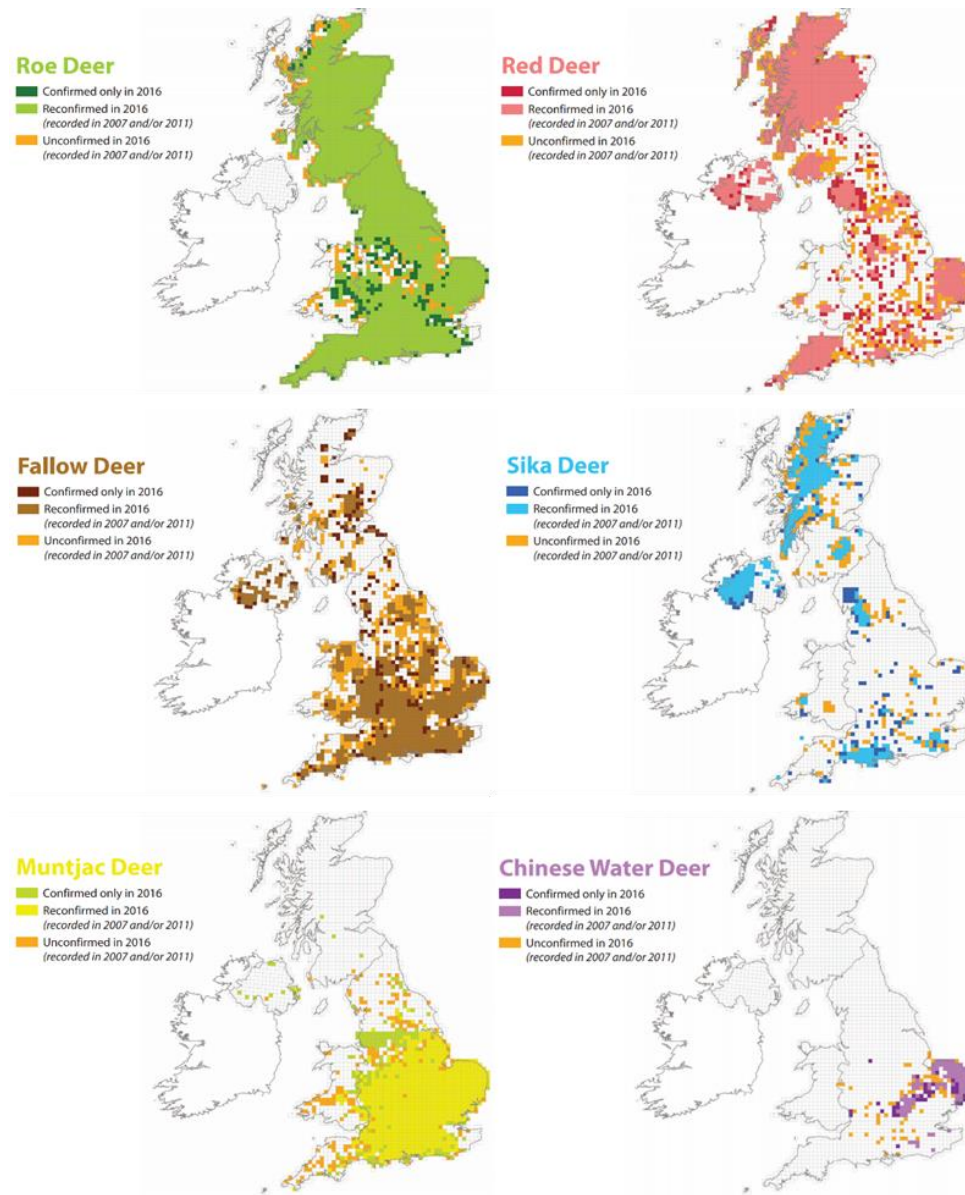


Figure 2-1: Distribution of British deer species in the 2016 Deer Distribution survey by the British Deer Society.

Adapted from [<https://www.bds.org.uk/science-research/deer-surveys/deer-distribution-survey/>]

2.2 Materials and methods

2.2.1 *Contributions to the manuscript*

Sample collection, DNA preparation and sequencing of BDS samples from British deer species was performed by Amy Robinson. Existing archived DNA samples of UK deer (red, roe and sika deer) were shared by Karis Baker (Durham University), Josephine Pemberton and Sílvia Pérez-Espona (The University of Edinburgh), Czech red deer samples from Jarmila Krojerová-Prokešová (Czech Academy of Sciences, Czech Republic) and were sequenced by Helen Williamson. Norwegian red deer samples were prepared and sequenced by Mariella E. Güere and Helene Tharaldsen (Norwegian Veterinary Institute, Oslo, Norway). Analysis and manuscript preparation by Amy Robinson.

2.2.2 *Sample overview*

For the analysis of *PRNP* variation in British cervids, a total of 1003 samples were analysed either from existing DNA archives, or tissue samples collected by the British Deer Society (BDS). This included 480 red deer (388 archived DNA samples, 92 BDS collected tissue samples), 297 roe deer (171 archived, 126 BDS), 83 sika (73 archived and 10 BDS), 9 sika-red hybrids (archived), 66 fallow deer (BDS), 41 muntjac (BDS) and 27 Chinese water deer (24 BDS and 3 archive). Exotic deer DNA samples were provided by the National Museum of Scotland (17 archived samples) and the Scottish deer centre (2 frozen muscle samples). Reindeer samples were collected from live animals as nasal swabs (20 swabs).

2.2.3 *Sample collection*

Sample collection was organised and facilitated through the British Deer Society (BDS). Members of the BDS collected samples on a voluntary basis during usual stalking activities from red, roe, fallow, sika, muntjac and Chinese water deer on a voluntary basis. Sampling was targeted to areas and/or species not well

represented by available DNA archives (section 2.2.1). Tissue sampling kits were distributed by post and contained a 30ml Plastic Universal tube containing 10ml of 20% DMSO in saturated NaCl. Each individual tube was placed into a mailing container with absorbent lining (Sarstedt 78.897). 5 tubes were sent in each kit, which also contained an absorbent sheet (Airsea 1031) packaged into a UN338 compatible mailing box (Sarstedt 95.903). Each sample was tagged with a specific 'TGGSN' number and the following data recorded: sample location, age, sex, grid reference (if possible) and date. To collect samples from live animals, Performagene™ DNA collection kits (DNAGenotek) were distributed, and the animal information recorded as above.

2.2.4 DNA extraction

Genomic DNA was extracted from approximately 1x2mm sections of ear-tip or frozen muscle tissue digested overnight with proteinase-K (PK) at 37°C, followed by standard phenol/chloroform purification, ethanol precipitation and re-suspension in 50µl 1XTE. DNA was extracted from Performagene™ DNA collection kits (DNAGenotek) as per manufacturer's instructions.

2.2.5 PRNP Polymerase Chain Reaction (PCR)

PCR reactions were prepared with a master mix containing template DNA, primers, dH₂O and AmpliTaq Gold® 360 Master Mix (Thermo Fisher Scientific)

Table 2-1: Standard PCR reaction for amplification of PRNP

	Volume for 1 reaction (µl)
DNA template	1*
Sterile dH ₂ O	23.6*
2X AmpliTaq gold 360 Master Mix (Thermo Fisher Scientific)	25
Forward primer (100µM stock)	0.2
Reverse primer (100µM stock)	0.2
Total: 50µl	

* Variable volume of DNA was used (1-5µl) depending on concentration, and sterile dH₂O decreased accordingly to give a 50µl total.

(Table 2-1). The optimal primer pairs were determined for each species and are detailed in tables 1 and 2. PCR conditions were as follows: PCR conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 10 min. An annealing temperature of 61 °C was used for all primers listed in Table 2-2.

2.2.6 Primer design

Primers used in the sequencing of the cervid *PRNP* ORF are listed in Table 2-2, and the successful primer pairs for each species detailed in Table 2-3. Primers were designed against the red deer ORF, with a minimum GC content of 50%, and were synthesised by Sigma-Aldrich.

Table 2-2: Primers for the open reading frame (ORF) of cervid PRNP

Primer sequences

DeerPrP-143d (Forward)
ATGGAATGTGAAGAACATTTATGACCTA
DeerPrP-213d (Forward)
AGGTCAACTTTGTCCTTGGAGGAG
DeerPrP+139u (Reverse)
TAAGCGCCAAGGGTATTAGCAT
DeerPrP-AR1 (Reverse)
TCCCCAACCTGGTAAAGA

Table 2-3: Primer pairs effective for amplification of cervid PRNP by species

Deer species	Primer pairs
Red	DeerPrP-143d / DeerPrP+139u DeerPrP-213d / DeerPrP+139u
Roe	DeerPrP-143d / DeerPrP+139u DeerPrP-213d / DeerPrP+139u
Sika	DeerPrP-143d / DeerPrP+139u DeerPrP-213d / DeerPrP+139u
Fallow	DeerPrP-213d / DeerPrP-AR3 DeerPrP-213d / DeerPrP-AR2 DeerPrP-213d / DeerPrP+139u
Muntjac	DeerPrP-213d / DeerPrP-AR2
Chinese Water deer	DeerPrP-213d / DeerPrP-AR3 DeerPrP-213d / DeerPrP-AR2 DeerPrP-213d / DeerPrP+139u DeerPrP-213d / DeerPrP+70u
Reindeer	DeerPrP-143d / DeerPrP-AR3 DeerPrP-213d / DeerPrP+70u

Primer pairs shown in bold were most effective for each species. Other primer pairs were variably successful.

2.2.7 Agarose gel electrophoresis

To visualise PCR products, 15µl of the reactions were run on a 1.5% agarose gel [1.5% w/v agarose in 1X TAE (diluted 10X stock solution, 0.89M Tris, 0.89 orthoboric acid, 0.2M EDTA) and 0.1µl/ml SYBRsafe® (Life Technologies). The gel was run at 170V for 30 minutes and visualised under UV light.

2.2.8 Activated charcoal purification of PCR products

The remaining 35µl PCR reaction was purified with addition of 15µl 1.6%w/v activated charcoal in dH₂O, vortexed briefly, incubated for at least 10 minutes, and centrifuged at 700 x g for 5 minutes to pellet charcoal.

2.2.9 Sequencing of PRNP

Purified PCR fragments were sequenced directly using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) as per manufacturer's instructions. The reverse primer DeerPrP+70u (Table 2-2) was used for sequencing all deer species. Sequencing reactions were further purified by the addition of EDTA and ethanol before loading onto an Applied Biosystems 3130 Genetic Analyser for Sanger sequencing. Alternatively, sequencing reactions were sent directly to Edinburgh Genomics for Sanger sequencing. Sequence data were analysed using Chromas and DNASTar Lasergene version 14.

Table 2-4: Sequencing reaction mix using the BigDye Terminator v3.1 Cycle Sequencing Kit

	Volume for 1 reaction (µl)
PCR template	2*
Sterile dH ₂ O	5*
BigDye Ready Reaction Mix	1
5X sequencing buffer	1.5
Primer: DeerPrP+70u 10µM (100µM stock diluted 1:10)	0.5
Total: 10µl	

*PCR template may be varied by increasing/decreasing sterile dH₂O volume such that total volume is 10µl.

RESEARCH ARTICLE

Open Access



Variation in the prion protein gene (*PRNP*) sequence of wild deer in Great Britain and mainland Europe

Amy L. Robinson^{1*} , Helen Williamson¹, Mariella E. Güere², Helene Tharaldsen², Karis Baker³, Stephanie L. Smith⁴, Sílvia Pérez-Espona^{1,4}, Jarmila Krojerová-Prokešová^{5,6}, Josephine M. Pemberton⁷, Wilfred Goldmann¹ and Fiona Houston¹

Abstract

Susceptibility to prion diseases is largely determined by the sequence of the prion protein gene (*PRNP*), which encodes the prion protein (PrP). The recent emergence of chronic wasting disease (CWD) in Europe has highlighted the need to investigate *PRNP* gene diversity in European deer species, to better predict their susceptibility to CWD. Here we report a large genetic survey of six British deer species, including red (*Cervus elaphus*), sika (*Cervus nippon*), roe (*Capreolus capreolus*), fallow (*Dama dama*), muntjac (*Muntiacus reevesii*), and Chinese water deer (*Hydropotes inermis*), which establishes *PRNP* haplotype and genotype frequencies. Two smaller data sets from red deer in Norway and the Czech Republic are also included for comparison. Overall red deer show the most *PRNP* variation, with non-synonymous/coding polymorphisms at codons 98, 168, 226 and 247, which vary markedly in frequency between different regions. Polymorphisms P168S and I247L were only found in Scottish and Czech populations, respectively. T98A was found in all populations except Norway and the south of England. Significant regional differences in genotype frequencies were observed within both British and European red deer populations. Other deer species showed less variation, particularly roe and fallow deer, in which identical *PRNP* gene sequences were found in all individuals analysed. Based on comparison with *PRNP* sequences of North American cervids affected by CWD and limited experimental challenge data, these results suggest that a high proportion of wild deer in Great Britain may be susceptible to CWD.

Introduction

Chronic wasting disease (CWD) is a prion disease of cervid species that is widespread in North America and has recently emerged in Europe. It belongs to a family of diseases termed transmissible spongiform encephalopathies (TSEs), which cause progressive and invariably fatal neurodegenerative disorders in humans and animals. CWD was first described in the 1960s as a wasting syndrome of captive mule deer (*Odocoileus hemionus hemionus*) and black-tailed deer (*Odocoileus hemionus columbianus*) in Colorado wildlife facilities [1]. It has since been identified

in North America in wapiti (elk, *Cervus canadensis*) white-tailed deer (*Odocoileus virginianus*), moose (*Alces alces*) and a single captive red deer (*Cervus elaphus*) [2, 3]. It is currently the only animal prion disease recognised in both captive and wild populations, and efficient transmission has led to spread of the disease across North America. At the time of writing, CWD is present in at least 25 US states and three Canadian provinces [4], as well in South Korea, introduced via importation of infected wapiti [5].

Member States of the European Union (EU) and Norway were believed to be free of CWD based on a survey conducted between 2006 and 2009 on 3274 farmed and 10 049 wild deer [6], with the vast majority being red deer (10 110). However, the first European case of CWD, and the first natural case in a reindeer (*Rangifer tarandus*) was identified in Norway in March 2016 [7].

*Correspondence: a.robinson@ed.ac.uk

¹ Division of Infection and Immunity, The Roslin Institute and The Royal Dick School of Veterinary Studies, University of Edinburgh, Midlothian EH259RG, UK

Full list of author information is available at the end of the article



Subsequent culling of the affected reindeer population resulted in detection of another 18 infected animals, and increased surveillance in Scandinavia has identified further “atypical” cases in three moose and a single red deer in separated locations in Norway, and individual moose in Finland and Sweden. Although the origins of CWD in Scandinavia are uncertain, the wide geographic distribution of cases raises concern that disease may have been present and undetected for some time [8–11].

The development of prion diseases is associated with misfolding of the prion protein (PrP^C) into a protease-resistant form (PrP^{Sc}). The sequence of the open reading frame (ORF) of the gene encoding the prion protein (*PRNP*) is strongly associated with susceptibility to prion diseases. This association has been exploited in breeding programmes such as the National Scrapie Plan in the UK, which selectively bred for disease resistant *PRNP* genotypes to reduce the incidence of classical scrapie in sheep [12, 13]. Cervid *PRNP* genetics have been widely studied in North America, where both natural and experimental infection of deer species has allowed for the identification of *PRNP* polymorphisms that are associated with reduced incidence of disease and/or slower disease progression. The first identified was M132L in wapiti, which encodes a methionine to leucine change at codon 132. Genotype surveys of CWD affected deer populations have produced conflicting evidence regarding the association of codon 132 variation with incidence of disease [14, 15], but CWD challenges of deer [16, 17] and transgenic mice [18] suggest that the 132L variant reduces susceptibility to infection. Other important *PRNP* polymorphisms associated with CWD susceptibility include S225F in mule deer, [19–22], Q95H, G96S and A116G in white-tailed deer [23–28]. Although many of these *PRNP* polymorphisms have been associated with reduced susceptibility to CWD, none of them appear to confer complete resistance.

Experimental transmissions of CWD provide some evidence of susceptibility in deer species found in Europe. CWD has been transmitted orally to red deer [29], reindeer [30] and Reeves’ muntjac deer [31, 32]. Fallow deer have been infected via intracerebral inoculation [33], but failed to become infected when co-grazed with infected mule deer [34]. However, due to small sample sizes in all these studies, it is difficult to draw associations between susceptibility to experimental CWD infection and host *PRNP* genotype. It is also not known if the *PRNP* genotypes of the experimental animals used in these North American studies are representative of the genetics of native wild European deer populations.

The extent of *PRNP* sequence variation in European deer species has not previously been studied in detail,

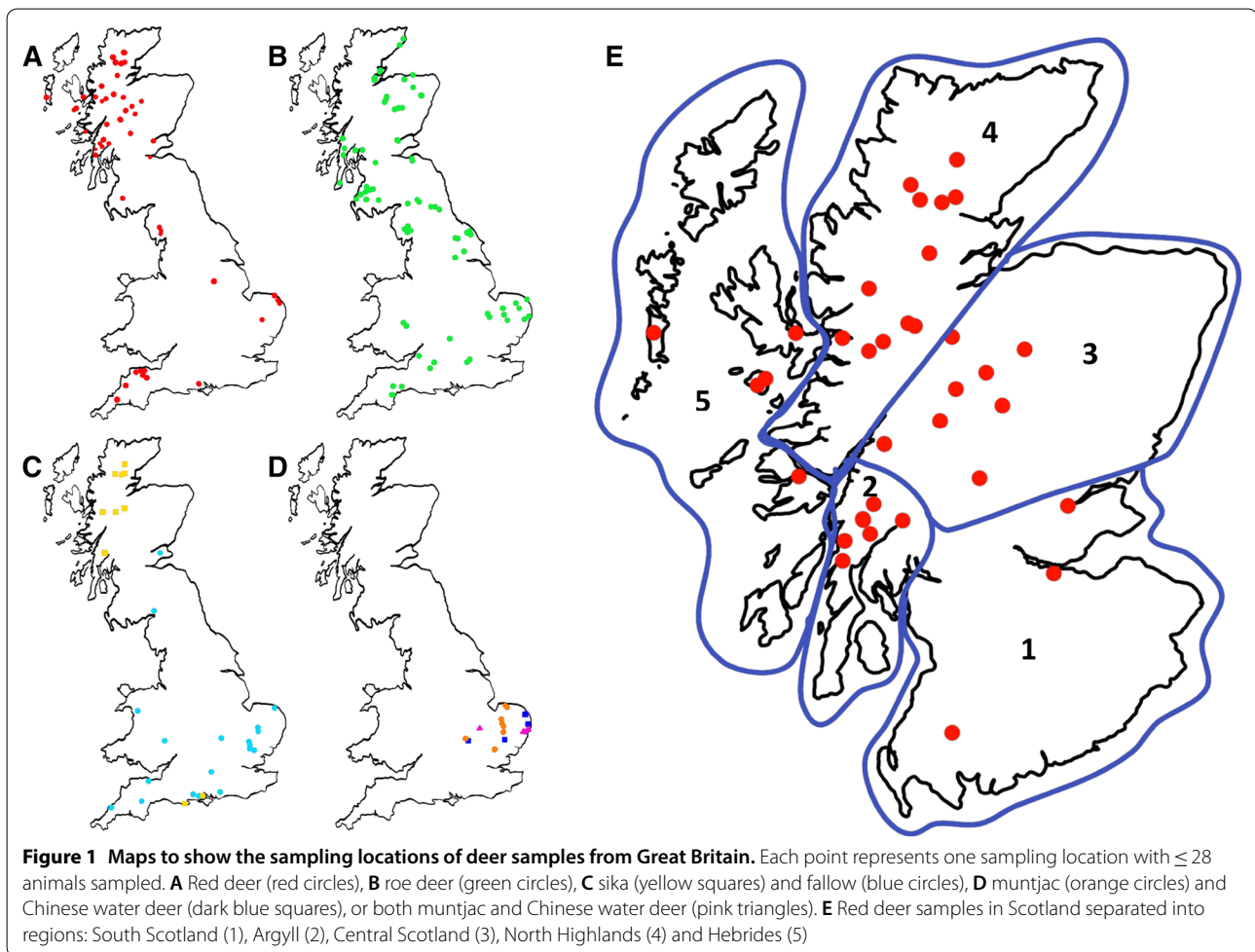
and this genetic information will be critically important in estimating potential susceptibility of these populations to emerging CWD and informing risk assessment and control/surveillance strategies. Novel *PRNP* variants (not seen in North American cervids) that show evidence of association with reduced susceptibility or resistance to CWD have the potential to be used in breeding programmes for captive and farmed deer. The primary aims of this study were (i) to perform a comprehensive survey of the protein-coding sequence of the *PRNP* gene in the six major free-ranging or wild species of deer found in Great Britain (GB) and (ii) to compare the *PRNP* genotype distributions in British and European populations of red deer, as red deer are one of the most numerous wild species in both GB and mainland Europe, and are also an economically important game species due to revenue generated via hunting, tourism and venison production [35].

Materials and methods

Samples from British deer species

A total of 1003 samples collected from wild deer in GB (England, Scotland and Wales) were analysed. Archived DNA samples were provided by JP, SPE and SLS for red deer, sika deer, and red/sika hybrids, and by KB for roe deer. The procedures for sample collection and genomic DNA preparation for these archives have been described previously [36–38]. Additional samples were collected by stalkers in the British Deer Society to give representation of geographical areas and species not covered by the existing archives. These included samples collected during routine culling, and samples collected from culled deer at a meat processing facility. Samples consisted of small sections of ear tips, which were preserved in 20% DMSO/saturated NaCl solution during transportation/storage [39]. For each sample, further information including the sex, estimated age, and postcode/grid coordinates of the location of the animal, were recorded. The distribution of sampling sites for each deer species are shown in Figure 1.

In total, samples from 480 red deer were analysed, of which 388 were from the archived collection, and 92 samples were collected by BDS deer stalkers. Samples from 83 sika deer were analysed, 73 samples were archived DNA, and 10 stalker collected samples from the South of England. All samples collected by members of the BDS were identified by animal phenotype only, whereas archived DNA samples of red and sika deer were selected both on phenotypical appearance and microsatellite genotyping data [38, 40], whereby a *Q* value of *Q*=1 indicated a pure red deer, *Q*=0 indicated a pure sika deer. Nine sika-red hybrids were also included in this study and were classified as having $0.091 \leq Q \leq 0.772$.



For analysis red deer populations were separated into the following regions: Southern England (mainly Exmoor National Park) ($n=78$), Northern England (Lake District) ($n=32$), Southern Scotland ($n=23$), Argyll ($n=47$), Central Highlands ($n=142$), Northern Highlands ($n=114$) and Hebrides ($n=44$) (Figure 1).

A total of 297 samples from roe deer were analysed, 171 from a DNA archive [37], and 126 collected by BDS deer stalkers. Samples from 66 fallow deer and 41 muntjac deer were collected by BDS deer stalkers. A total of 27 Chinese Water deer samples were analysed, 3 from a DNA archive [41] and 24 supplied by BDS deer stalkers.

Samples from other European red deer

Genotype analysis was also performed on 50 wild red deer collected from six counties in Norway, and 66 archived DNA samples from wild red deer ($n=46$) and wild sika ($n=20$) collected in five regions of the Czech Republic [42, 43]. In central Europe, 2 distinct lineages of red deer are present. Of the 46 Czech red deer, 24 were of Western lineage, and 22 of Eastern lineage.

Extraction and purification of genomic DNA

Genomic DNA was extracted from small sections of ear-tip tissue, digested with proteinase K (PK) at 37 °C overnight, followed by standard phenol/chloroform purification, ethanol precipitation and re-suspension in 1XTE buffer, as described previously [44]. Norwegian samples were prepared from brain tissue using DNeasy® Blood & Tissue Kit (Qiagen, Oslo, Norway) according to manufacturer's instructions.

PRNP gene amplification and sequencing: British and Czech samples

The ORF of cervid *PRNP* (771 bp) was amplified by PCR using AmpliTaq Gold 360 master mix (Thermo Fisher Scientific) and primer pairs –143d (ATGGAATGTGAA GAACATTTATGACCTA) or –213d (AGGTCAACT TTGTCCTTGAGGAG) with +139u (TAAGCGCCA AGGGTATTAGCAT) or AR2 (GCAAGAAATGAG ACACCACCAC) for British and Czech red, roe and sika deer, –213d and AR3 (ACCACTACAGGGCTGCAG GTA) for fallow and Chinese water deer, and –213d and

AR2 (GCAAGAAATGAGACACCACCAC) for muntjac deer. PCR conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 1 min, and a final 72 °C for 10 min. PCR fragments were sequenced by sanger sequencing using primer +70u (GCTGCAGGTAGATACTCCCTC) and BigDye® reagents (Life Technologies, Paisley, UK) as recommended by the manufacturer. Sequence data were analysed using Chromas and DNASTar Lasergene version 14.

PRNP gene amplification and sequencing: Norwegian samples

The ORF was amplified using PCR primers Ce19_F (ATT TTGCAGATAAGTCATC) and Ce 778_R (AGAAGATAATGAAAACAGGAAG) [14]. PCR conditions were 95 °C for 2 min, followed by 36 cycles at 95 °C for 30 s, 51 °C for 30 s and 72 °C for 45 s, then a final cycle at 72 °C for 10 min. PCR fragments were directly sequenced using Ce19_F and Ce 778_R and BigDye® reagents (Life Technologies, Paisley, UK) as recommended by the manufacturer. Sequence data were analysed using Seqscape v3.0 software, Sequence Scanner v2.0 (Applied Biosystems) and MEGA7 version 7.0.26 [45].

Statistical analysis

The Chi square test was used to compare haplotype frequencies between different regions and countries.

Accession numbers

Sequences were deposited in Genbank with the following accession numbers: *Capreolus capreolus* MK103016; *Dama dama* MK103017; *Cervus nippon* MK103018, MK103019; *Muntiacus reevesi* MK103020–MK103023; *Hydropotes inermis* MK103024–MK103026; *Cervus elaphus* with 247L MK103027.

Results

The ORF encoded by exon 3 of the *PRNP* gene is highly conserved among cervid species, and non-synonymous polymorphisms identified in individual species are usually reported as variations from a consensus sequence [25]. Following this convention, the amino acid substitutions resulting from *PRNP* sequence polymorphisms identified in the deer species examined in our survey are summarized in Table 1.

British red deer

Four single nucleotide polymorphisms (SNPs) were identified in 480 British red deer samples: a synonymous SNP at codon 136 (t/c, nucleotide position 408), and three non-synonymous polymorphisms at codons 98

(a/g, nucleotide position 292), 168 (c/t, nucleotide position 502) and 226 (g/c, nucleotide position 676) giving rise to amino acid changes threonine to alanine (T98A), proline to serine (P168S) and glutamine to glutamic acid (Q226E), respectively. Linkage was found between positions 408 and 676 (codons 136 and 226), such that the haplotypes were either 408t-676c or 408c-676g. The SNPs resulting in substitutions at positions 98 and 168 occurred exclusively on a Q226 background and the following four haplotypes were inferred: T₉₈-P₁₆₈-E₂₂₆ (TPE), T₉₈-P₁₆₈-Q₂₂₆ (TPQ), A₉₈-P₁₆₈-Q₂₂₆ (APQ) and A₉₈-S₁₆₈-Q₂₂₆ (ASQ).

Norwegian and Czech red deer

Only the TPE and TPQ haplotypes were identified in Norwegian red deer. The synonymous codon 136 polymorphism was observed in the same haplotype linkage as described above. Czech red deer had TPE, TPQ and APQ haplotypes, as well as a novel non-synonymous SNP at codon 247 (a/c nucleotide position 739) leading to a conservative isoleucine to leucine substitution which occurred in linkage with APQ (APQ-L₂₄₇).

British sika, Czech sika and sika-red deer hybrids

Scottish pure sika ($n=73$), Czech sika ($n=20$), English sika ($n=8/10$) were all homozygous for the TPQ haplotype. Both British and Czech sika had a synonymous polymorphism at codon 133 (g/c, position 399) at frequencies of 0.08% and 0.1% respectively. Of the nine genetically determined sika-red hybrids from Argyll ($0.091 \leq Q \leq 0.772$) three were homozygous TPQ/TPQ, four were heterozygous TPE/TPQ, and two were heterozygous for the APQ haplotype (APQ/TPQ and APQ/TPE). The two animals heterozygous for the APQ haplotype were characterised as red deer-like ($Q=0.71, 0.74$), as were two of four TPE/TPQ heterozygotes ($Q=0.77, 0.62$). Of the 10 English animals identified as sika by phenotype only, two were TPE/TPQ heterozygous and were therefore possibly sika-like hybrids.

Roe and fallow deer

The *PRNP* ORF of British roe deer ($n=297$) all encoded the haplotype described above as TPQ. Roe deer were monomorphic in *PRNP* sequence, with no variation in nucleotide sequence in any of the animals analysed. Likewise, fallow deer ($n=66$) showed no variation in *PRNP* sequence and encoded the TPE haplotype with an additional amino acid substitution of asparagine for serine at codon 138 (TPE-N₁₃₈).

Table 1 Amino acid variation within the ORF of cervid *PRNP* in the surveyed deer species

	<i>PRNP</i> codon number					
	98	100	138	168	226	247
Consensus sequence	T	S	S	P	Q	I
Red <i>Cervus elaphus</i>						
TPQ	–	–	–	–	–	–
TPE	–	–	–	–	E	–
APQ	A	–	–	–	–	–
APQ-L ₂₄₇	A	–	–	–	–	L
ASQ	A	–	–	S	–	–
Roe <i>Capreolus capreolus</i>						
TPQ	–	–	–	–	–	–
Sika <i>Cervus nippon</i>						
TPQ	–	–	–	–	–	–
Fallow <i>Dama dama</i>						
TPE-N ₁₃₈	–	–	N	–	E	–
Muntjac <i>Muntiacus reevesii</i>						
SPQ	S	–	–	–	–	–
Chinese water deer						
<i>Hydropotes inermis</i>						
TPQ	–	–	–	–	–	–
TPQ-N ₁₀₀	–	N	–	–	–	–

Muntjac and Chinese water deer

The *PRNP* ORF of all 41 muntjac deer analysed encoded serine at position 98, allowing their *PRNP* haplotype to be described as S₉₈P₁₆₈Q₂₂₆ (SPQ). In addition, synonymous polymorphisms were identified at nucleotide positions 15 (c/t), 126 (g/a) and 606 (c/t) at varying frequencies. The *PRNP* coding region of 27 Chinese water deer revealed two amino acid sequence polymorphisms; a serine to asparagine substitution at codon 100 (g/a, nucleotide position 299) and a 24 bp deletion of the octapeptide repeat (ccccatggaggtggctggggtcag) which corresponds to the in-frame loss of the amino acid sequence PHGGGWGQ. In most species, a glycine-rich peptide sequence (octapeptide repeat) is present in five consecutive copies in the N terminal region of wild type PrP^C, whilst Chinese water deer had a four octapeptide repeat haplotype. From the genotypes it was inferred that the deletion was linked to 100S (100S-Δ_{71–78}). The haplotype frequencies were 0.21 for 100S, 0.25 for 100SΔ_{71–78}, and 0.54 for 100 N. Seven animals were heterozygous for 100SΔ_{71–78}, whilst three were homozygous S₁₀₀Δ_{71–78}/S₁₀₀Δ_{71–78}. The most common genotype in this sampling was N₁₀₀/N₁₀₀ (0.35).

Red deer genotype and haplotype frequencies and distributions

Red deer showed the greatest variation in *PRNP* sequence amongst the six deer species surveyed, with four

haplotypes identified in the British population. Regional variation was evident within Great Britain in the distribution and frequency of the four *PRNP* haplotypes and resulting genotypes (Tables 2 and 3). For the TPQ and TPE haplotypes, frequencies in different regions of Scotland and England ranged from 0.11 to 0.62 and 0.31 to 0.88 respectively. The difference was most striking in Southern England, which had a very high frequency (0.88) of the TPE haplotype, whereas in Northern England, the TPQ haplotype was predominant (0.67). The APQ and ASQ haplotypes were found almost exclusively in Scottish red deer, with only one heterozygous TPE/APQ individual identified in Northern England. The frequencies of APQ and ASQ haplotypes showed regional variation within Scotland, with the highest frequencies seen in the Central Highlands, Argyll and Southern Scotland (Table 3). When compared using the Chi squared test, the Central Highlands, Lowlands and Argyll were significantly different in haplotype frequency when compared to the Northern Highlands and Hebrides ($P < 0.05$), as were the overall haplotype frequencies of England and Scotland ($P < 0.05$).

The haplotype frequencies in GB were also compared by Chi squared test with those in red deer from Norway and the Czech Republic, as well as previously published surveys of Scotland, Italy and Spain [46, 47] (Table 4). The number of samples was too low to assess the extent of regional variation within Norway and the Czech

Table 2 Genotype frequencies of *PRNP* polymorphisms in British red deer populations

98	168	226	England			Scotland					
			Total	S	N	Total	SS	A	CH	NH	H
TT	PP	EE	58 (64)	78 (61)	9 (3)	29 (107)	31 (7)	28 (13)	13 (19)	41 (47)	48 (21)
TT	PP	QE	26 (28)	19 (15)	41 (13)	28 (102)	22 (5)	15 (7)	28 (39)	34 (39)	27 (12)
TT	PP	QQ	15 (17)	3 (2)	47 (15)	10 (36)	9 (2)	8 (4)	11 (15)	10 (11)	9 (4)
TA	PP	QE	1 (1)	–	3 (1)	14 (52)	26 (6)	19 (9)	16 (23)	9 (10)	9 (4)
TA	PP	QQ	–	–	–	8 (31)	4 (1)	13 (6)	11 (16)	5 (6)	5 (2)
AA	PP	QQ	–	–	–	3 (13)	–	11 (5)	4 (6)	1 (1)	2 (1)
TA	PS	QE	–	–	–	4 (14)	4 (1)	4 (2)	8 (11)	–	–
TA	PS	QQ	–	–	–	2 (8)	4 (1)	2 (1)	4 (6)	–	–
AA	PS	QQ	–	–	–	2 (7)	–	–	5 (7)	–	–
AA	SS	QQ	–	–	–	–	–	–	–	–	–
			100 (110)	100 (78)	100 (32)	100 (370)	100 (23)	100 (47)	100 (142)	100 (114)	100 (44)

S: Southern England, N: Northern England, SS: Southern Scotland, A: Argyll, CH: Central Highlands, NH: Northern Highlands, H: Hebrides.

Percentage frequencies are given with animal numbers in brackets.

Table 3 Haplotype frequencies of *PRNP* polymorphisms in British red deer populations

Haplotype	Southern England	Northern England	Southern Scotland	Argyll	Central Highlands	Northern Highlands	Hebrides	GB average
TPE	0.88	0.31	0.57	0.47	0.39	0.63	0.66	0.56
TPQ	0.12	0.67	0.24	0.23	0.32	0.29	0.25	0.29
APQ	0	0.02	0.15	0.27	0.20	0.08	0.09	0.12
ASQ	0	0	0.04	0.03	0.09	0	0	0.03
Total	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Republic. In Norway, where only the TPQ and TPE haplotypes were identified, haplotype frequencies were very similar to those found in Southern England, i.e. 0.89 for TPE and 0.11 for TPQ. In the Czech Republic, frequencies of the TPQ and TPE haplotypes were approximately equal (0.35 and 0.44, respectively), while the APQ and APQ-L₂₄₇ haplotypes were present at frequencies of 0.16 and 0.05, respectively. The APQ-L₁₄₇ haplotype was only seen in the Czech red deer belonging to the Western lineage ($n=24$) [43]. The haplotype frequencies presented in Table 4 were compared across countries, and whilst most differences between countries were statistically significant ($P<0.05$), those in Spain and the Czech Republic were not.

Discussion

This study represents the largest survey to date of *PRNP* genetic variation in the wild (free-ranging) deer population of any European country and is the first to include free-ranging muntjac and Chinese water deer. The analysis included over 1003 animals from six different species of deer present in Britain, as well as red deer and sika

deer from mainland Europe. Ten haplotypes were identified, five of which were present in red deer. The majority of polymorphisms identified in the ORF (exon 3) of the *PRNP* gene have been reported previously [32, 46–49], apart from the I247L polymorphism in red deer from the Czech Republic, and the octapeptide repeat deletion in Chinese water deer. Deletion of the third glycine-rich octapeptide repeat is not an uncommon variation, having previously been described in chamois [47], lions [50], and some primates [51]. This is the first time a functional octapeptide deletion variant has been described in deer.

Red deer showed the greatest variation in *PRNP* sequence, with four haplotypes identified in Great Britain, designated TPQ, TPE, APQ and ASQ. The TPQ, TPE and APQ haplotypes have been previously reported in surveys of Italian, Scottish (as haplotypes 1, 8 and 10) [46] and Spanish red deer [47]. The ASQ haplotype was not identified in Italy and Spain, and only reported previously in a single Scottish red deer [46]. Non-synonymous polymorphisms previously identified in Italian red deer at codons 59 (G59S) and 208 (M208I) in single individuals [46, 52], as well as low frequency synonymous

Table 4 Comparison of haplotype frequencies across European red deer populations

	TPE	TPQ	APQ	ASQ	Total animals	References
England	0.71	0.28	0 ^a	0	110	
Scotland	0.52	0.29	0.16	0.04	370	
Scotland	0.69	0.27	0.05	0	132	[46]
Norway	0.89	0.11	0	0	50	
Czech	0.35	0.44	0.21 ^b	0	46	
Spain	0.36	0.43	0.21	0	209	[47]
Italy	0.28	0.62	0.10	0	191	[46]

^a A single animal was identified with the APQ genotype.

^b The L₂₄₇ polymorphism was present on an APQ background at a frequency of 0.05.

polymorphisms at codons 15, 21, 59, 78 and 79 [46] were not present in our surveyed populations.

Taking our results together with previously published surveys, the total number of European red deer analysed for *PRNP* sequence variation stands at 1108 individuals. In every region or country surveyed, TPQ and TPE haplotypes were present at the highest frequencies (Tables 3 and 4). In comparison to other European red deer populations, there was a high frequency of the TPE haplotype in South England (predominantly the Exmoor National Park) and in Norway, which is possibly due to historical population declines of red deer with refugial populations in these areas [53]. The APQ haplotype was absent from both Southern England and Norway but was found at similar frequencies in Scottish (0.16), Czech (0.16), Spanish (0.21) and Italian (0.10) red deer [46, 47]. In our study, it was evident that the frequency of the APQ haplotype showed marked variation between different Scottish regions (Table 3). This may explain why our frequency of the APQ haplotype was higher (0.16) than the previous Scottish survey (0.05); in the previous study, samples were collected from the Isle of Rum and an unspecified location on mainland Scotland, which may not have coincided with regions where we identified the highest APQ haplotype frequencies. The ASQ and APQ-L₂₄₇ haplotypes were present at frequencies of 0.04 and 0.05 in Scottish and Czech red deer populations respectively and may be unique to these populations. Our results emphasize that there are large regional variations in *PRNP* genotype frequencies in British red deer populations, and similar variation in genotype frequencies between the red deer populations surveyed in European countries to date. Regional variations in the genetics of Scottish red deer populations have been previously reported, in part due to natural barriers [36] and in part due to introductions of non-native red deer into Scotland from England and further afield [54].

In contrast to red deer, our roe deer samples showed no variation in their *PRNP* sequence. Whilst the

number of analysed animals across studies stands now at 541, only one synonymous change at codon 24 has previously been reported in two individuals [48]. Considering that roe deer are now the most widespread species in the UK, it is possible that this lack of diversity may be due to a population bottleneck following the near-extinction of British roe deer prior to the 1800 s [37]. However, a similar lack of *PRNP* diversity was also evident in European roe deer populations from Italy [46], Scandinavia [48] and Spain [47]. Similarly, we found little evidence of sequence variation in sika, fallow and muntjac deer, with the result that each of these species is predicted to express a single specific variant of the PrP protein. However, with a relatively small number of animals analysed (103, 92 and 41 respectively), less frequent polymorphisms may still be found in larger surveys. The *PRNP* sequence we identified in British fallow deer (described here as TPE-N₁₃₈), was identical to previously published sequences from Scandinavia [48] and Spain [47], as well as fallow deer previously used in experimental challenge experiments [34, 55]. Our data support the conclusion that the N₁₃₈ change is fixed in the fallow deer sequence and not polymorphic. The three haplotypes identified in 27 wild Chinese water deer is more than might be expected considering that the UK population was established relatively recently from small numbers of escapees from captive collections.

Comparison of the *PRNP* genotypes in European deer with those in North American cervid species with European data can be used to help predict their potential susceptibility to CWD, although confirmation of these predictions under natural or experimental challenge is essential. The TPQ haplotype, which we identified at high frequencies in British red (0.29), Roe (1.0), Sika (1.0) and Chinese Water deer (0.21), is identical to *PRNP* sequences found in North American cervids that are highly susceptible to CWD. Our analysis, and published data to date for European deer species, has not

identified any *PRNP* polymorphisms that are associated with reduced CWD susceptibility in North American cervids, e.g. M132L, G96S [25]. This suggests that a relatively high proportion of free-ranging/wild deer in Britain and Europe may be susceptible to CWD, or at least to the predominant strains of CWD in North America.

The association of non-synonymous *PRNP* polymorphisms found only in British/European deer with susceptibility to CWD has yet to be determined. Amino acid substitutions at codon 98 are found in red (T98A) and muntjac (T98S) deer, and nearby codons 95 and 96 play a role in CWD susceptibility in white-tailed deer [24]. However, muntjac deer with identical *PRNP* sequences can be experimentally infected with North American CWD isolates [31] and recently camels, which encode alanine at the equivalent position, were reported to develop natural prion disease [56].

The substitution of serine for proline at codon 168 in red deer is of particular interest, as this position is located in the $\beta 2$ - $\alpha 2$ loop of the prion protein, an area critical for protein structure and stability [57]. The murine prion protein with a serine substitution at the equivalent residue showed no difference to wildtype in efficiency of conversion to PrP^{Sc} in an in vitro cell-free conversion assay [57]. However, in sheep the substitution of leucine for proline at codon 168 was associated with very prolonged incubation periods for experimental BSE infection [58], and a reduction of cell-free conversion efficiencies [59]. The ASQ haplotype seen in red deer has not been tested in vitro or by prion challenge, so any potential association with prion disease is still unresolved.

The novel *PRNP* haplotype TPQ-L₂₄₇ identified in Czech red deer may be less likely to influence susceptibility as it represents a conservative amino acid substitution within the C-terminal signal peptide which is cleaved from the mature prion protein [60], however it is possible that it may have unknown effects that influence pathogenesis.

In North America codon 226 encodes either E (e.g. wapiti) or Q (e.g. mule deer and white-tailed deer), whereas in European red deer both variants are present and heterozygous animals are common. Codon 226 is in the third α -helix of PrP^C, a part of the protein involved in pathogenic processes during prion disease. Nearby polymorphisms include S225F, which is associated with reduced CWD susceptibility in mule deer [20], and Q222K, which is strongly associated with prion disease resistance in goats [61, 62]. Studies in transgenic mice expressing cervid PrP with Q226 or E226 suggest that variation at 226 may contribute to differences in disease progression and susceptibility to certain prion strains [63, 64]. Following experimental

oral transmission of CWD to four deer of EE₂₂₆, QQ₂₂₆ and EQ₂₂₆ genotypes, all animals became infected [65]. The small sample size of this study does not allow a definite conclusion regarding the association of codon 226 with CWD susceptibility, or the effect of heterozygosity at this position.

The TPQ-N₁₀₀ haplotype identified in Chinese water deer has not been reported in any other cervid but N₁₀₀ is common to other mammalian prion proteins including bank vole, a species highly susceptible to CWD challenges [66]. The fact that fallow deer failed to become infected with CWD when exposed to infected mule deer for up to 7 years suggests that the substitution of asparagine at codon 138 may be associated with relative resistance to infection, but it is possible that other species-specific genetic factors also play a role [34].

In summary, this study has provided an analysis of *PRNP* sequence variation in the six major deer species found in the wild in Great Britain and adds to our knowledge of *PRNP* variation in European deer species in general. Based on a comparison of the sequences with those found in North American cervids affected by CWD, it appears likely that a large proportion of the British deer population would be susceptible to CWD. The effect of specific *PRNP* haplotypes only found in European species, such as ASQ in Scottish red deer, on CWD susceptibility are still to be determined.

Abbreviations

CWD: chronic wasting disease; *PRNP*: prion protein gene; SNP: single nucleotide polymorphism; EU: European Union; GB: Great Britain; ORF: open reading frame.

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Authors' contributions

Study design and manuscript writing: ALR, JMP, WG, FH; sample collection and DNA preparation: ALR, HW, MEG, HT, KB, SLS, SP-E, JK-P; sample analysis: ALR, HW, MEG, HT, WG. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Division of Infection and Immunity, The Roslin Institute and The Royal Dick School of Veterinary Studies, University of Edinburgh, Midlothian EH259RG, UK. ² Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Oslo, Norway. ³ Department of Biosciences, Durham University, South Road, Durham DH1 3LE, UK. ⁴ The Royal Dick School of Veterinary Studies, University of Edinburgh, Midlothian EH259RG, UK. ⁵ Institute of Vertebrate Biology of the Czech Academy of Sciences, Květná 8, 603 65 Brno, Czech Republic. ⁶ Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic. ⁷ Institute of Evolutionary Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, UK.

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2.3 Supplementary results

2.3.1 *PRNP variation in exotic deer species in the UK*

There are a number of cervid species in zoos and wildlife parks across the UK. In order to extend our study on the wild UK deer populations to include these exotic species, we utilised an archive of tissue samples from the National Museum of Scotland, tissue samples from the Scottish Deer Centre, and nasal swabs from live reindeer. The reindeer sampled included animals from the Cairngorms (see introduction), as well as captive reindeer from various locations in England. We sampled 33 animals including 20 reindeer, three Pere David deer, two Thorold's deer, two Visayan Water deer, and one each of Chital, Bawean Hog, Indian Hog, Bactrian, Pudu and Barasingha deer (Table 2-5). Whilst these numbers are relatively small, this work includes some species where published sequences are not available.

Analysis of the non-synonymous substitutions in exotic deer revealed a number of sequence variants and polymorphic sites. The substitution of asparagine at codon 138 previously identified in fallow deer (138N, 413G>A 414C>T) was also identified in Visayan spotted deer (138N, 413G>A), and Barasingha, Pere David and Indian Hog deer were all heterozygous at this position (138S/N). Interestingly, these three 138N heterozygous deer also displayed substitutions at codon 208 (208I, 624 G>A) and were either heterozygous (Indian Hog deer, Pere David deer), or homozygous (Barasingha) for this change.

Cervids differed at codon 226, as has been previously reported for elk and deer in North America, and as we have documented in British deer populations.

Analysis of the cervid species presented in Table 2-5 as well as the published *PRNP* variation in European and North American cervid species [290, 311] demonstrates that the amino acid at codon 226 is dependent on deer subfamily. The family Cervidae is divided into two subfamilies, *cervinae* (Old world) and *capreolinae* (New world). Old world deer species include the genera *Cervus* (including the Pere David's deer *Elaphurus davidianus*), *Axis*, *Dama*, and *Rucervus*. New world deer species include the genera *Capreolus*, *Hydropotes*,

Alces and Odocoileini (*Rangifer* + American genera)[364]. Old world deer may express either 226Q or 226E, whereas new world deer express exclusively 226Q (based on available species and sequences). The substitution at codon 243 (243M, 727G>A) in Pudu and at codon 254 (254T, 761T>C) in Visayan spotted deer have not been previously reported in cervid species. The substitution at codon 249 (249V, 745T>G) has recently been identified in Chital deer [365] and was also present in the deer we analysed. All of these substitutions are in the extreme C-terminal part of the protein (GPI anchor signal sequence). An octapeptide repeat variation similar to that present in Chinese water deer was identified in one of the two Visayan spotted deer analysed. The animal was heterozygous for the deletion, which combined with a short sequence read meant that analysis of the full ORF of this animal was not possible.

The reindeer analysed showed a level of *PRNP* variation consistent with what has previously been reported in Norwegian populations [310], and previously published alleles (A,B, D and E) are shown in Table 2-5. The nasal swabs used for sampling on analysis of live animals produced inconsistent results, with 6 of 26 animals sampled not suitable for analysis due to either poor DNA yield and/or poor sequencing results. Sequencing of reindeer produced 8/20 animals with partial sequences suitable for analysis (170-771bp), however due to the previously published linkages for allele E [310], it was possible to infer results at codon 2 for these animals. Four alleles were identified in my analysis (Table 2-5), which are made up of polymorphisms at codons 2 (V2M, 4G>A), 129 (G129S, 385G>A), 169 (V169M, 505G>A), 176 (N176D, 526A>G) and 225 (S225Y, 674C>A). Allele A (225S) and allele E (2M.129S.169M) were most common, both with frequencies of 0.4. Allele B (225Y) and allele D (176D) were less common, with frequencies of 0.05 and 0.15 respectively. Due to the small sample sizes it is difficult to compare data between reindeer sampled in Scotland (Cairngorms) (n=8) and in England (n=12), however alleles A,E and D were identified in Cairngorm reindeer, whilst A,B,E and D were identified in English reindeer. Allele C, encoding a 5/4 octapeptide repeat variation was not identified in our analysis.

Table 2-5: Amino acid variation in the PRNP sequence of exotic cervid species.

Species	Consensus	PRNP codon number											Freq	Ref
		2	129	138	169	176	208	225	226	243	249	254		
		V	G	S	V	N	M	S	Q	V	F	I		
Chital (<i>Axis axis</i>)	2	-	-	-	-	-	-	-	E	-	V	-	1	
Bawean Hog deer (<i>Hyelaphus kuhlii</i>)	2	-	-	-	-	-	-	-	-	-	-	-	1	TPQ
Bactrian (<i>Cervus elaphus bactrianus</i>)	2	-	-	-	-	-	-	-	E	-	-	-	1	TPE
Indian hog deer (<i>Axis porcinus</i>)	1	-	-	-	-	-	-	-	E	-	-	-	0.5	
	1	-	-	N	-	-	I	-	E	-	V	-	0.5 ¹	
Visayan Spotted (<i>Rusa alfredi</i>)	3	-	-	N	-	-	-	-	-	-	-	T	0.75	
	1	-	-	N	-	-	-	-	-	-	-	T	0.25 ²	5/4del
Thorold's (<i>Cervus albirostris</i>)	2	-	-	-	-	-	-	-	-	-	-	-	0.5	TPQ
	2	-	-	-	-	-	-	-	E	-	-	-	0.5	TPE
Pudu (<i>Pudu puda</i>)	2	-	-	-	-	-	-	-	-	M	-	-	1	
Barasingha (<i>Rucervus duvaucelii</i>)	1	-	-	N	-	-	I	-	E	-	-	-	0.5	
	1	-	-	-	-	-	I	-	E	-	-	-	0.5	
Pere David (<i>Elaphurus davidianus</i>)	5	-	-	N	-	-	-	-	-	-	-	-	0.83	
	1	-	-	-	-	-	I	-	E	-	-	-	0.17	
Reindeer (<i>Rangifer Tarandus</i>)	16	-	-	-	-	-	-	-	-	-	-	-	0.4	Allele A
	2	-	-	-	-	-	-	Y	-	-	-	-	0.05	Allele B
	6	-	-	-	-	D	-	-	-	-	-	-	0.15	Allele D
	16	M	S	-	M	-	-	-	-	-	-	-	0.4	Allele E
Total: 66														

Consensus amino acids are given at the top, and variations from this denoted in the table. Full coding sequences were analysed except where noted, and in reindeer: 16/40 had partial sequences (170-771nt) only, however alleles were inferred due to previously published linkages.

¹The linkage between 138N and 208I in Indian hog deer could not be determined. ²Partial (193-316nt) only, sequence is uncertain and predicted based on available data. Amino acids: V, valine; M, methionine; G, glycine; S, Serine; N, asparagine; D, aspartate; I, isoleucine; Y, tyrosine; Q, glutamine; E, glutamate; F, phenylalanine; T, threonine.

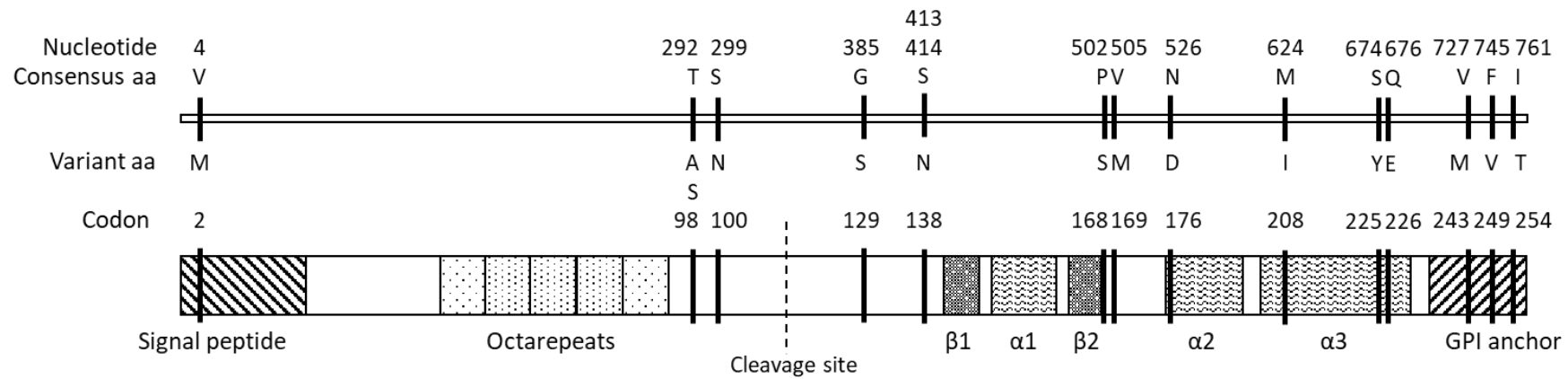


Figure 2-2: Non-synonymous variation in the PRNP ORF of British wild and exotic cervid species.

Consensus and variant amino acids are shown with numbered nucleotide and amino acid codons, mapped to structural domains within the prion protein.

2.4 Discussion

Following the first detection of CWD in Europe in 2016, the susceptibility of European cervid populations has been a key area of interest. Red deer are known to be susceptible, following the report of an atypical case of CWD in a single Norwegian red deer [345], CWD cases in farmed red deer in North America [366, 367] and experimental transmission of CWD to this species [368]. The Norwegian case was in an animal of the 226EE (TPE/TPE) genotype, and both experimental transmissions and cases in farmed deer were TPQ and TPE homozygous or heterozygous animals (Gordon Mitchell, pers. comm). Cases have not been reported in animals expressing the polymorphic variants described at codons 98 or 168, and the effect of these polymorphisms on susceptibility is unknown. Experimental oral CWD transmissions in muntjac [206] and sika [369], expressing sequences consistent with what we have observed in wild populations for these species, demonstrate their likely susceptibility to CWD. Furthermore, infected individuals of these species showed clinical and pathological characteristics consistent with North American CWD cases. Reindeer have likewise been experimentally infected with CWD, either by intracranial or oral inoculation, or by co-housing with infected reindeer [241, 242]. Expression of the 138N polymorphism is associated with reduced susceptibility of reindeer to CWD when orally infected [241]. However, reindeer expressing the 138N allele develop CWD after intracranial inoculation with CWD from white-tailed deer, mule deer and elk. Reindeer expressing the 138N allele were also infected via direct contact or indirectly through the environment, though PrP^{Sc} was detectable only in lymphoid tissues of these animals. Interestingly, the extent of lymphoreticular PrP^{Sc} deposition was significantly lower in 138SN animals [242]. Gene-targeted mice expressing 138NN-cervid PrP did not develop clinical disease (up to ±660dpi) when inoculated intracranially with various CWD isolates, whilst 138SS were positive for disease at ±450dpi [311]. Together, these results suggest that the 138N polymorphism has a significant impact on CWD prion propagation.

Fallow deer are homozygous for asparagine (N) at codon 138, whereas North American reindeer populations are polymorphic at codon 138, and the 138N allele has not been identified in Norwegian reindeer populations [307–310]. I have also demonstrated the presence of the 138N substitution in a Visayan spotted deer (homozygous), and Bawean hog, Barasingha, and Pere David's deer (heterozygous). Fallow deer have been shown to succumb to CWD following intracranial inoculation [243, 244], albeit following extended incubation periods, however appear resistant to more natural routes of infection [245]. This may be due to the 138N substitution, which is the only position at which fallow deer differ from susceptible North American species.

In cervid species where there have been no reported natural cases and no experimental transmissions, comparison of the *PRNP* gene can provide useful insight into potential susceptibility. A similar approach has also been used to predict the susceptibility of various carnivore species to prion disease [64]. The evidence presented in this chapter supports the finding that the *PRNP* gene is well conserved in cervid species, and it is likely that there is broad susceptibility to CWD across cervid species. This was also the conclusion of a recent phylogenetic analysis of Cervidae [370]. Where the *PRNP* varies from the susceptible consensus or wild-type sequence, polymorphisms are almost always associated with reduced susceptibility rather than full resistance to disease. RT-QuIC evaluation of potential susceptibility for commonly identified and hypothetical cervid *PRNP* sequences found that rate of amplification aligned best with *in-vivo* susceptibilities. Using this metric the wild-type white-tailed deer sequence 95Q/96G/116A/226Q (QGAQ) generally had higher amplification rates, whereas alleles associated with resistance *in-vivo* had lower amplification (including 138N in fallow deer) [301]. The evolutionary basis for the geographical distribution and the varying frequencies of the various *PRNP* polymorphisms identified in different cervid populations is unknown. It is possible that they represent recent, random anomalies in the *PRNP* gene, or perhaps they are an indication that some cervid populations once faced

primordial prion strains [301]. It is likewise unclear if the expression of rarer *PRNP* alleles is associated with reduced reproductive fitness, explaining their rarity in cervid populations. This may be the case for mule deer expressing 225FF, which displayed subtle atypical traits [233]. Whilst this chapter primarily focuses on the non-synonymous variation observed in *PRNP*, synonymous polymorphisms and other host factors as well as strain, route and dose all play a role [317]. Genetic variants outwith the *PRNP* ORF in regulatory regions may impact *PRNP* expression levels and have been associated with BSE susceptibility in cattle [371]. However, a similar analysis in Rocky mountain elk failed to identify any associations between CWD susceptibility and variants after accounting for the M132L polymorphism, which cut odds of CWD by more than half [372].

A number of polymorphisms that may impact susceptibility to CWD have been identified in cervid species described in this chapter (Figure 2-2).

Polymorphisms identified in red deer are of particular interest as they are a common species throughout Europe, are economically important and are also a farmed species, which opens the possibility for selective breeding for CWD resistance. However, even in North America where cervid *PRNP* genetics are well characterised, breeding for alleles with reduced susceptibility is not widely practiced and has additional considerations due to the uncertain effects of certain *PRNP* genotypes on prolonged infectious shedding and the emergence of new prion strains (section 1.6.5.3). The P168S polymorphism in Scottish red deer is of particular interest as it is located in the $\beta 2$ - $\alpha 2$ loop region of the prion protein. The $\beta 2$ - $\alpha 2$ loop is a critical structure in the prion protein encompassing residues 168-178 and is well-defined and rigid in cervid species [142]. Amino acid sequence variants in this region of the prion protein are a key determinant in protein structure and cross-species transmission (section 1.4.3). Additionally, a polymorphism at codon 168 in sheep, P168L, is protective against BSE, with animals heterozygous for ARQ-168L surviving three times longer than ARQ-168P homozygotes [373]. The P168L polymorphism also reduces the cell-free conversion efficiency of ovine recombinant PrP induced by three different prion

sources [75]. It would therefore be interesting to perform further experiments to determine the effect of the P168S polymorphism in red deer on CWD susceptibility.

Two polymorphisms (V196M and N176D) identified in reindeer also occur in this loop region [306, 310]. Interestingly, neither of these polymorphisms (alleles D and E) were detected amongst Norwegian CWD cases, but accounted for 20.8% of the alleles in negative Nordfjella reindeer [310]. Additionally, reindeer heterozygous for allele E (2M.129S.169M) failed to develop CWD after oral challenge [241] and had prolonged incubation periods following intracranial inoculation [252].

Recent investigations have highlighted the interaction of the β 2- α 2 loop with the distal region of the third α -helix (α 3), forming a solvent-accessible discontinuous epitope [312]. Stabilising long-range interactions between the loop and α 3 occur in rabbit PrP, a species generally regarded as resistant to prion infection [374]. Molecular dynamics simulations have shown that similar long range stabilising interactions occur between aspartate at residue 170 (170D) and tyrosine at residue 228 (228Y) in deer expressing PrP-F225 (associated with resistance in mule deer), but not in deer expressing wild-type deer or elk PrP structures, allowing hydrogen bonding between side-chains of these residues and increased stability of this discontinuous epitope [304]. A similar effect may therefore be caused by the S225Y polymorphism in reindeer, which could explain why this allele (allele B, Table 2-5) was less frequent in Norwegian CWD cases [310]. Together, these results reinforce the key importance of the amino acid sequence of the loop region and the third α -helix and the potential for polymorphisms in these areas to have profound impact upon CWD susceptibility.

Also in the third α -helix is the residue at which North American deer and elk species differ, expressing either 226Q or 226E respectively. Studies in transgenic and gene-targeted mice expressing either 226Q or 226E alleles have suggested that differences at residue 226 alter the structure of PrP^C, conversion

to PrP^{Sc} and controls the manifestation of CWD strains [149, 160, 229, 320]. Analysis of wild British deer, exotic deer species and comparison to the published *PRNP* sequences for cervids shows that the residue at 226 is partly defined by Cervidae subfamily (section 2.3.1); this variation within cervid species may therefore contribute to the variety of CWD strains. An additional polymorphism in the third α -helix ($\alpha 3$) was identified in Barasingha, Indian hog and Pere David's deer. The presence of either methionine or isoleucine at codon 208 (M208I) has previously only been reported in a single red deer [375]. This polymorphism has not been identified in our study of British, Czech and Norwegian red deer, or in other surveys of red deer *PRNP*, suggesting that it is an uncommon variant [336, 355]. Whilst the consensus amino acid expressed in cervid species at codon 208 is methionine, the substitution to isoleucine mirrors the consensus amino acid in sheep. Studies using rec-PrP based PMCA have shown that sheep rec-PrP with substitutions to introduce the cervid rigid loop (S173N and N117T) blocked the ability of recombinant PrP to propagate prion seeds (scrapie, BSE, CWD). However this effect was abrogated by the addition of methionine at codon 208, and sheep rec-PrP with amino acid substitutions 177T and 208M (with or without 173N) was highly permissive to misfolding by all PrP^{Sc} seeds [141]. These results further support the importance of long-range interactions between the $\beta 2$ - $\alpha 2$ loop and the third α -helix, and the importance of the amino acid residues in these areas.

I have also identified a number of extreme C-terminal variants in cervid *PRNP*, including V243M in pudu, I247L in red deer, F249V in Chital and Indian hog deer, and I254T in Visayan spotted deer. The F249V substitution in deer has also recently been reported in wild Chital deer (n=133) from Texas and Hawaii, which were all monomorphic [365]. Whether or not any of these substitutions have an effect on misfolding is unknown, as they are present in the GPI-anchor signal sequence (GPI-SS) that is cleaved during normal post-translational processing of the prion protein (Figure 2-2). Whilst these substitutions are therefore unlikely to have an effect on the conformation of the mature protein, a study utilising scrapie infected neuroblastoma cells has shown that mutations in

the GPI-SS can influence both GPI anchoring and conversion efficiency of PrP [376].

Analysis of the *PRNP* genetics of Norwegian Nordfjella reindeer identified 5 alleles, two of which significantly increased the odds of CWD infection (alleles A and C) [310]. Considering the origins of reindeer in the UK, it is likely that much of their genetic variation mirrors what is seen in Northern European reindeer, as was reflected in our identification of 4 of the 5 alleles described. Allele C, which encodes a 5/4 octapeptide deletion in reindeer was not identified, though this may be due to the low numbers of animals sequenced. Alleles A (40%) and E (40%) were equally frequent, whereas in the Nordfjella population allele A was most frequent (46.3%) and allele E was relatively rare (5.8%)[310]. However, our sample population is small, and the frequencies of the alleles identified in the Nordfjella population may differ from those in other Norwegian reindeer populations. The North American reindeer polymorphism S138N (413G>A) was not present in Norwegian Nordfjella reindeer population and likewise was not identified in our samples [241, 307, 308, 310].

Based on the genetic analyses presented in this chapter, and comparison with published materials, it is likely that a significant proportion of European cervids would be susceptible to at least the strains of CWD circulating in North America. However, there are a number of polymorphisms identified at important sites in the protein structure which have unknown effects on CWD susceptibility and warrant further study. Utilising *in-vitro* conversion assays can highlight variants which have a significant impact on protein structure, stability or conversion, which may then be further analysed *in-vivo*, for example through transgenic mouse bioassay. Understanding the genetic variation of European cervid populations and predicting their potential susceptibility to CWD will be of vital importance for risk management and modelling of CWD spread in Europe. The work presented in this chapter provides surveys of British, Czech and Norwegian wild deer populations including some exotic cervid species, which will likely be followed by similar surveys in other European countries, completing our picture of *PRNP* variation in European cervid populations.

3 Developing an *in-vitro* conversion assay to assess the effects of *PRNP* genetic variation on chronic wasting disease susceptibility

3.1 Introduction

This chapter describes the work undertaken to develop an *in-vitro* assay for the assessment of the effect of non-synonymous cervid *PRNP* polymorphisms on the *in-vitro* replication of CWD prions, to better predict the effect of genetic changes in European cervid populations on disease susceptibility.

Much of the evidence regarding genetic susceptibility of cervid species to CWD has been determined in North America, where genetic surveys reveal the prevalence of CWD in animals with different *PRNP* genotypes (reviewed in [290] and section 1.6.5). However, in cases where the *PRNP* allele is rare, or in areas where the disease is newly emerging, these types of studies may not be possible. The ideal experiment to investigate the effects of *PRNP* polymorphisms on disease susceptibility would be to experimentally challenge animals of the correct species with known genotypes. Such experiments have provided evidence of the effects of *PRNP* variation on CWD in North America [226, 233, 234, 236, 237, 241, 245]. However, these experiments are extremely expensive, require the use of large animals not well suited to laboratory conditions, take many years to complete, and often have very low sample sizes which can make the effects of genotype difficult to determine. To overcome some of these issues, transgenic (Tg) mice that are engineered to express physiological levels (gene-targeted) or overexpress *PRNP* from a variety of species, are commonly used in prion research. Experimental challenge of transgenic mice expressing specific *PRNP* genotypes can provide evidence of the effects of different *PRNP* polymorphisms [160, 304, 318, 328]. However, such techniques still rely upon experimental animals which limits sample sizes, and experiments are lengthy and expensive to perform. Assays modelling the conversion of the cellular form of the prion protein to the misfolded form are therefore a valuable alternative for assessing the effects of *PRNP* polymorphisms. The efficiency of conversion is closely associated with homology between the amino acid sequence of the infecting PrP^{Sc} and the host cellular PrP, which is why *PRNP* genetics often play a large part in susceptibility. Protein misfolding cyclic amplification (PMCA) and

real-time quaking induced conversion (RT-QuIC), have been developed over the last 15 years (section 1.3.5), and have been used for the ultra-sensitive detection of PrP^{Sc}, the de-novo generation of prion infectivity and to model susceptibility, strain properties and cross-species transmission. [279]. PMCA utilises sonication to facilitate amplification, whereas RT-QuIC instead uses intermittent shaking, however both techniques rely upon a normal cellular form of the prion protein to be used as 'substrate' for conversion by a small amount of infectious PrP^{Sc} 'seed'. Avoiding the use of experimental animals, the production of PrP^C substrate may be achieved through protein expression in bacterial systems or in mammalian tissue culture. The former involves the expression of recombinant prion protein (rPrP) in bacteria (most often *E.coli*) followed by a series of purification and refolding steps [377]. Recombinant prion protein can also be used to investigate the effects of amino acid substitutions on protein structure via various protein modelling techniques (such as NMR) or use in a fibrillisation assay which measures the propensity of a protein to misfold without the addition of an infectious seed (PrP^{Sc}) [142, 312, 378, 379]. Equally, the rPrP substrate can be utilised in *in-vitro* prion replication assays such as the cell-free conversion assay [73–75], or real-time quaking induced conversion (RT-QuIC) [301, 302]. Expression of recombinant prion protein has several advantages, it is relatively fast and has the potential to produce a variety of different PrP sequence variants, and when optimised, protein can be produced in large amounts. RT-QuIC estimation of chronic wasting disease susceptibility in North American species has been achieved using various naturally occurring and hypothetical PrP variants [301]. However, bacterially expressed proteins lack post-translational modifications, such as glycosylation and the GPI anchor, which would be present in the mammalian cell. There is also more opportunity for sequence difference to influence conversion in PMCA, compared to RT-QuIC, as in the latter only the C-terminal residues (~160 to 231) refold to give a PK-resistant amyloid core (and corresponding increase in ThT fluorescence), whereas cell-free conversion and PMCA reactions rely on immunoblotting and detection of larger PK-resistant cores consisting of residues from ~90-231 equivalent to those seen *in-vivo* [96,

380]. This may be why RT-QuIC assays tend to be less constrained by sequence differences between seed and substrate, with poor amplification of prion diseases in substrate from the same species, whereas bank-vole substrate appears to provide almost universal amplification [95, 96, 381].

The protein misfolding cyclic amplification assay (PMCA) was developed using brain homogenate as the source of substrate PrP^C [77]. Transgenic mouse brains are most commonly used, and provide a physiologically accurate substrate, some flexibility in *PRNP* genotype, and the ability to perform short-term experiments with a variety of PrP^{Sc} inocula or 'seeds'. However this approach is limited by the availability of transgenic mouse lines, and also requires the use of experimental animals to provide material. The expression of PrP^C in mammalian cell line provides flexibility in the *PRNP* sequence expressed, and cell lysates have been used as a PMCA substrate which can be scaled up as required. PrP^C expressing cell cultures have many uses in prion research; cell culture studies have been used to investigate the effects of *PRNP* glycosylation mutants [382], and the ability to infect PrP^C expressing cell cultures to study prion propagation has been widely studied [383–387]. A variety of cell types, both neuronal such as N2a and GT1 [388–390], and non-neuronal such as mouse fibroblasts [391] and rabbit kidney epithelial cells (RK13) transfected to express *PRNP* are capable of propagating prions. RK13 cells have proved to be the most adaptable, as the exogenous expression of mouse, bank vole, sheep or cervid protein has been achieved, with the possibility of propagating prion disease from these species [384, 392–394]. RK13 cells also have the advantage that they do not express endogenous PrP^C, and therefore can be transfected directly with the *PRNP* of interest. Whilst various transfection methods have been used to generate PrP^C expression in RK13 cells, the methods broadly fall into 1) transient expression [140, 395] or stable expression [384, 392, 396]. As well as providing the opportunity to perform cell infection studies, cell lysates may be utilised in cell-free conversion assays, or as a PMCA substrate [79, 140, 386, 395, 397, 398]. Transient expression of prion protein in RK13 cells has recently been used by Kurt *et al* [395] to generate a large number of variants based on

mouse, human, bank vole or rabbit *PRNP* sequences, and test their amplification in PMCA using a number of different prion seeds including CWD. This approach allowed identification of residues that markedly impacted upon prion conversion and potential for cross-species transmission.

This chapter details work undertaken towards developing an *in-vitro* assay, initially to assess the effects of novel polymorphisms identified in British red deer described in the previous chapter, and the 138N substitution identified in fallow deer which may be associated with reduced susceptibility [243–245]. We also hoped to produce a flexible and easily modifiable system which could be reproduced to assess the effect of novel polymorphisms which may be detected in other European cervid populations, or other species.

3.2 Materials and methods

3.2.1 *Molecular biology techniques*

3.2.1.1 *Vectors for the expression of cervid PrP in bacteria*

Glycerol stocks for four North American cervid *PRNP* variants in Rosetta DE3 pLysS bacteria transformed with pTrcHis vector were provided by Andrew Gill (The Roslin Institute, Edinburgh). Two variants were white-tailed deer *PRNP* with variants 96G (QGAS) and 96S (QSAS), and two variants were Rocky Mountain Elk *PRNP* with variants 132M (GMSE) and 132L (GLSE). British cervid *PRNP* variants including red deer APQ, ASQ and fallow deer were generated via mutagenesis as described in Table 3-1, and 3.2.1.3.

3.2.1.2 *Vectors for the expression of cervid PrP in mammalian cells*

The pcDNA3.1+ vector for mammalian expression (or bacterial) was synthesised by Genscript to contain full-length red deer (TPQ) open reading frame. Alternative vectors were also obtained: pTREtight2 (gifted by Markus Ralser (Addgene plasmid # 19407, pTET-ON (gifted by Didier Vilette (National Research Institute for Agriculture, Food and Environment (INRAE), France) and linear puromycin resistance marker (Takara Bio, 631626). The pTREtight2 vector was delivered in DH5alpha competent *E.coli* as a bacterial stab, whereas the pTet-On plasmid was provided purified and was then transformed into DH5alpha (library efficiency) cells (3.2.1.6). Bacteria transformed with the plasmid vectors were grown up to create glycerol stocks (3.2.1.5) and plasmid DNA was purified (3.2.1.4) for restriction enzyme digestion to confirm plasmid identity (3.2.4.1).

3.2.1.3 *Mutagenesis reactions*

The Quikchange II mutagenesis kit (Agilent, 200523) was used as per manufacturer's instructions. Mutagenesis oligos were designed using the online tool available at [<https://www.agilent.com/store/primerDesignProgram.jsp>] and were synthesised and HPLC purified by Sigma-Aldrich. The plasmid DNA

stocks used as a dsDNA template are shown in Table 3-1. Mutagenesis PCR was carried out as follows: (1) 95°C for 30s, (2) 95°C for 30s, (3) 55°C for 1 min, (4) 68°C for 4.24 mins (pTrcHis) or 68°C for 5.4 mins (pcDNA3.1+) (1 min per kb length), with (2-4) repeated 15 times. Mutagenesis reactions were digested with Dpn1 (10U) and transformed into XL1-Blue supercompetent cells as per manufacturer's instructions with a substitution of SOC media (Invitrogen™, 15544034) for NZY media. Single colonies were picked from overnight growths and grown up in 10ml LB broth before plasmid preparation as described (3.2.1.4).

Desired mutation	Plasmid template	Mutagenesis primers	Primer sequence
T98A (Red)	QGAS (pTrcHis)	Red98A_fwd Red98A_rev	CTGGGGTCAAGGTGGTGCCACAGTCAGTG CACTGACTGTGGGCACCACCTTGACCCAG
S138N (Fallow)	GMSE (pTrcHis)	Fallow138N_fwd Fallow138N_rev	GCTACATGCTGGGAAGTGCCATGAATAGGCCTCTTATAC GTATAAGAGGCCTATTCATGGCACTCCAGCATGTAGC
P168S (Red)	T98A (pTrcHis)	Red168S_fwd Red168S_rev	CCAACCAAGTGTACTACAGGTCAGTGGATCAGTATAATAAC GTTATTATACTGATCCACTGACCTGTAGTACACTTGGTTGG
3F4 epitope	pcDNA3.1 +_TPQ	AR_3F4_fwd AR_3F4_rev	CAGCTCCTGCCATATGCTTCATGTTGGTTTTTGGTT AACCAAAAACCAACATGAAGCATATGGCAGGAGCTG

Table 3-1: Site-directed mutagenesis reactions performed to generate British PRNP variants and the 3F4 epitope.

3.2.1.4 Colony selection, bacterial growth, and plasmid preparation

LB agar or broth was prepared with a final concentration of 100µg/ml ampicillin. Bacterial glycerol stocks were incubated on dry ice, streaked onto LB agar plates using sterile bacterial loops, and grown overnight at 37°C. Bacterial colonies were picked using a sterile pipette tip under flame, transferred to 8mls of LB broth and incubated with shaking at 37°C, 200rpm overnight. The bacterial broth was then used in preparation of glycerol stocks (3.2.1.5), or for the preparation of plasmid DNA. For molecular biology work, the QIAprep Spin miniprep kit (Qiagen, 27104) was used as per manufacturer's instructions. To produce endotoxin-free transfection-grade plasmid for cell culture work, the Purelink Hipure midiprep plasmid kit (Invitrogen, K210004) was used as per manufacturer's instructions.

3.2.1.5 Glycerol stocks

100µl of overnight bacterial culture was added to 10ml LB/amp broth, and incubated at 37°C, with shaking at 200rpm for 4 hours. 500µl of culture was added to 500µl of 30% glycerol/LB in an 1.5ml Eppendorf tube. Glycerol stocks were snap frozen in dry ice and stored at -80°C.

3.2.1.6 Bacterial transformation

Library Efficiency™ DH5α Competent cells (Invitrogen, 18263012), Rosetta™ 2(DE3) PlyS (Sigma-Aldrich ,71403-3) or XL1-Blue supercompetent cells were thawed on ice, mixed, and 50µl aliquoted into chilled 14ml round bottom falcon tubes (BD Biosciences, 352059). DNA was added (~20-100ng) and reactions were incubated on ice for 30 minutes, heat shocked for 45 seconds at 42°C, then placed on ice for 2 minutes. 0.5ml of SOC media (Invitrogen) was added and incubated for 1 hour at 37°C at 200rpm. 250µl was plated onto a LB agar plate (3.2.1.4) and incubated at 37°C overnight.

3.2.1.7 Sequencing of plasmids

Plasmids were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The pcDNA3.1+ vector was sequenced in both forward and reverse directions using AR_T7prom_fwd (TAATACGACTCACTATAGGG) and AR_BGH_rev (TAGAAGGCACAGTCGAGG) primers (Sigma-Aldrich). pTrcHis vectors were sequenced in both forward and reverse directions with pTrcHis forward (GAGGTATATATTAATGTATCG) and pTrcHis reverse (GATTTAATCTGTATCAGG) primers (Sigma-Aldrich). Sequencing reactions were carried out using the 'Seq_pro' program with (1) denaturation 96°C for 10 seconds, (2) annealing 53°C for 15 seconds (3) elongation at 60°C for 4 minutes, (2-3) repeated 25 times, and (5) hold at 4°C. Sequencing reactions were sent to Edinburgh Genomics for Sanger sequencing. Sequence data were analysed using Chromas (Technelysium Pty Ltd) and DNASTar Lasergene version 14.

Table 3-2: Sequencing reaction mix using the BigDye Termination v3.1 cycle sequencing kit

Component:	Volume for 1 reaction (μl)
Plasmid DNA	14 (1400ng)
Sterile dH ₂ O	0
BigDye Ready Reaction mix	2
5X sequencing buffer	3
Primer: pTrcHis Forward / pTrcHis Reverse	1
Total: 20μl*	

*Half-reactions with a total 10μl were also successful.

3.2.2 Production of recombinant cervid prion protein

3.2.2.1 Bacterial growth and induction of protein production

Bacteria were plated out from glycerol stocks and individual colonies selected for further growth. Colonies were grown overnight in 50ml LB broth with 100μg/ml ampicillin at 37°C and 225rpm. 10 ml of overnight culture was transferred to 500ml of terrific broth (TB) with 100μg/ml ampicillin and grown at 37°C and 225rpm until an OD₆₀₀ of 0.6-0.8 was reached. PrP protein expression was induced by addition of Isopropyl-β-d-thiogalactopyranoside (IPTG) to a final concentration of 1mM, followed by growth at 37°C and 225rpm for 16-18 hours. Bacteria were harvested by centrifugation of bacterial cultures at 22000 x g for 15 minutes at 4°C.

3.2.2.2 Bacterial lysis

Bacterial pellets were frozen at -80°C prior to resuspension in a lysis buffer [50mM Tris, 1mM EDTA, 100mM NaCl, pH8] at 9ml/g of cell pellet. Lysozyme (10mg/ml, 20μl per ml buffer) and PMSF (5mM, 2μl per ml buffer) were added and cultures incubated at 4°C with continuous stirring for 40 minutes. Solid sodium deoxycholate was added to a final concentration of 1mg/ml, and stirred for 30 minutes or until an increase in viscosity occurred. 5μl DNase (2mg/ml) was added per ml of lysis buffer and continuously stirred for up to 45 minutes, or until a decrease in viscosity occurred. Inclusion bodies were pelleted by centrifugation at 34,000 x g, 4°C for 15 minutes. Inclusion body pellets were stored at -80°C.

3.2.2.3 Purification of recombinant protein

Recombinant protein was purified for use in a fibrillation assay as described previously [377]. Briefly, inclusion body pellets were solubilised in IMAC buffer and prion protein purified from the supernatant using the intrinsic metal-binding properties of PrP. Purification was achieved by Immobilised Metal Affinity Chromatography (IMAC) on an AKTA™ FPLC system (GE Healthcare) using an IMAC column containing nickel-nitrilotriacetic acid (NiNTA) superflow resin (Qiagen) followed by desalting on a HiPrep™ 26/10 desalting column (GE Healthcare) and oxidative refolding overnight at 4°C in the presence of oxidised-glutathione. The protein was further purified by high performance liquid chromatography (HPLC) using a reverse-phase column (214TPC410-15, Vydac) eluting the protein with a linear gradient from water to acetonitrile acidified with 0.1% (v/v) trifluoroacetic acid. Protein concentration was measured using a Nanodrop™ (Thermo Fisher) and aliquots dried using a SpeedVac (Thermo Fisher) before storage at -80°C. To visualise protein purity in fractions following purification steps, 30µl of protein fraction with 1:10 β-mercaptoethanol in NuPAGE LDS sample buffer (4X) (Thermofisher) was run by NuPage gel electrophoresis and stained with instant blue™ (Sigma).

3.2.3 Fibrillation assay

The fibrillation assay was carried out as described previously [379]. Briefly, recPrP was solubilised in 6M Guanidine pH 6.0, and added to a reaction buffer such that the final concentration of recPrP was 5.2µM, with 2M guanidinium hydrochloride, 0.05M 2-(N-Morpholino)-ethanesulphonic acid (MES); pH 6.0, 0.01M thiourea, and 10µM thioflavin T (ThT). 160µl of recPrP in buffer (reaction master mix) and 3 Teflon balls (3/32" (2.381mm)) were added to each well in a 96-well plate, and the plate sealed. The plate was shaken at 900rpm, 37°C for 24 hours, with ThT fluorescence readings taken every 5 minutes at excitation 444nm and emission 485nm. Background fluorescence was calculated in triplicate from wells containing no rec-PrP and subtracted from the sample

readings. Sigmoidal curves were fitted to the data, and lag time (t_1) and rate of fibrillisation (K) calculated as described previously [398].

3.2.3.1 Maturation and PK digestion of fibrils

To confirm the presence of fibrils the reactions were dialysed in 10mM sodium acetate pH 5.0 and digested with proteinase K (PK) as described previously [379]. Proteins were visualised by NuPAGE electrophoresis (3.2.6) followed by silver staining using SilverQuest™ staining kit (Invitrogen) and imaging using a Gel-DOC (Bio-Rad).

3.2.4 Subcloning of cervid PrP insert into pTRETight2 vector

3.2.4.1 Restriction enzyme digests

Restriction enzymes BglII, NheI, XbaI, NcoI and BamHI (NEB) were used as per manufacturer's recommendations to perform restriction enzyme digests. Digests were run alongside the Trackit 1kb plus DNA ladder (Invitrogen) on an 0.8% agarose gel containing 0.1µl/ml SYBRsafe® (Life Technologies) in 1XTAE buffer (2.2.7). Gels were analysed and imaged on a UV transilluminator (NuGenius, Syngene).

3.2.4.2 Agarose gel extraction and purification

Digested vectors were visualised using a transilluminator (Safe Imager, Invitrogen) and cut from the agarose gel using sterile scalpel blades. The Monarch® DNA gel extraction kit (New England Biolabs) was used to purify selected bands according to manufacturer's instructions.

3.2.4.3 Ligation of new vector

Prior to ligation reactions, pTRETight2 was treated with shrimp alkaline phosphatase (rSAP) (New England Biolabs, M0371S) as per manufacturer's instructions, to prevent recircularization of the vector. Purified fragments of 783bp (*PRNP* insert flanked by restriction sites) and 2894bp (Empty backbone

of linearised pTRETight2 vector) were combined in either a 3:1 or 5:1 ratio calculated using the NEBioCalculator™ and the formula: required mass insert (g) = desired insert:vector molar ratio x mass of vector (g) x ratio of insert to vector lengths. T4 DNA ligase (New England Biolabs, M0202S) was used according to manufacturer's instructions. Ligated plasmid was transformed into DH5α as described in section 3.2.1.6, colonies selected, grown up and plasmid DNA prepared as described in section 3.2.1.4.

3.2.4.4 Diagnostic restriction digest and sequencing of new vectors

To check for insertion and correct orientation of the *PRNP* gene into the pTRETight2 vector, diagnostic restriction enzyme digests using BamHI and NcoI were carried out as described in section 3.3.6.2. Sequencing of vectors was undertaken using primer 961 (GGTGAAGTTCTCCCCCTTGGT) as described in section 3.2.1.7.

3.2.5 General cell culture methods

3.2.5.1 Cell lines and maintenance

Cell lines used during this study include a rabbit kidney epithelial cell line: RK13 (Rabbit kidney epithelial cells, 00021715-1VL, Sigma) and the Rov9 cell line (RK13 cells transfected with ovine VRQ *PRNP*) gifted by Didier Vilette (National Research Institute for Agriculture, Food and Environment (INRAE), France) Unless otherwise stated, immortalised cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (D5796, Sigma) containing 10% foetal bovine serum (FBS) (Gibco Fetal Bovine Serum, qualified, 10270106) v/v, with or without 1% Penicillin (100U/ml) and streptomycin (100µg/ml) solution. Cell work was carried out using aseptic technique. Cell cultures were maintained at 37°C, 0.5% CO₂ and varying between 50-100% confluence.

3.2.5.2 Freezing of cells

Cells were expanded and frozen to create either working cell banks for future experiments, or to create a master cell bank of newly created stable cell lines. Cells at <100% confluency were washed twice with PBS before addition of Trypsin-EDTA (1X) (Sigma) and incubation at 37°C for 5-10 minutes.

Dissociated cells were resuspended in DMEM media, spun at 800 x g or 3 minutes, and the media removed from the resulting cell pellet. The cell pellet was resuspended in freezing media (70% v/v DMEM, 20% v/v Foetal Bovine Serum, 10% v/v Dimethyl sulfoxide (DMSO) and 1ml aliquots were dispensed into cryotubes (Nunc™, Thermofisher; 4796843), and frozen at -80°C in a Mr Frosty (Nalgene®) according to manufacturer's instructions.

3.2.5.3 Thawing and splitting of cell cultures

Frozen cells were thawed quickly by addition 5mls of pre-warmed DMEM media, and cells pelleted by centrifugation at 800 x g for 2 minutes. Freezing media containing residual DMSO was removed and replaced with fresh supplemented DMEM. Cells were plated in an appropriate size surface treated cell culture dish (Nunc™, Thermofisher). When confluent, cells were split by removing media, washing 2 times in PBS (Gibco, 14190144), the addition of Trypsin-EDTA (1X) (Sigma) and incubation at 37°C for 5-10 minutes.

Dissociated cells were diluted (at least 10X) in media. Cells were replated at the desired concentration either by cell counting using a haemocytometer, or estimation by eye.

3.2.5.4 Lysis of cells

Cell culture media was removed from flasks and the cell monolayer washed twice in PBS. Cells were removed from the flask surface in ~1-10ml of chilled PBS using a cell scraper, keeping the flask over ice. PBS was added multiple times to ensure the collection of the majority of the cells, which were collected in a 50ml Falcon tube on ice. Cells were pelleted by centrifugation at 800 x g for

3 minutes, repeating if necessary. Cell pellets were resuspended in cold PMCA buffer (PBS, 1% Triton X100, 5mM EDTA, 150mM NaCl, 1 tablet cOmplete™ protease inhibitor tablet (Roche, 4693116001). Cells were lysed either by passage several times through 27g needles, or by sonication for five 30s periods, separated by at least 30 seconds on ice. Cell lysate was centrifuged at 2000 x g for 1 minute to pellet debris, and the supernatant collected and stored at -20°C before analysis.

3.2.5.5 Total protein concentration estimations of cell lysates

Total protein concentration was estimated via the BCA (Bicinchoninic Acid) method, using the Micro BCA™ protein assay kit (Thermo, 23235) according to the manufacturer's instructions. Briefly 1µl of cell lysate were prepared in 1ml of working reagent, which were compared to BSA protein standard curve. Samples were analysed by absorbance at 562nm on a spectrophotometer.

3.2.5.6 PNGase treatment of cell lysates

PNGase F (New England Biolabs, P0704) was used to remove N-linked glycans on PrP^C present in cell lysates and produce a single band on western blot. Reactions were undertaken as per manufacturer's instructions, followed by precipitation of proteins using cold acetone at -20°C for at least one hour before centrifugation at 15,600 x g for 15 minutes. Protein pellets were resuspended in sample buffer for western blot (see below).

3.2.6 Western blotting of cell lysates

Prior to electrophoresis, protein samples were denatured in NuPage™ LDS Sample Buffer (4x) (Invitrogen™, NP0008) supplemented with reducing agent (Invitrogen™, NP0009), at 95°C for 5 minutes. Samples were separated by electrophoresis on NuPage™ 4-12% Bis-Tris precast gels (Invitrogen, NP0336BOX). A Chameleon® 700 Pre-stained protein ladder (Licor Biosciences 928-70000) was used as a molecular marker spanning 8 to 160 kDa. Electrophoresis was carried out in NuPAGE™ MES running buffer (Invitrogen™,

NP0002), over a gradient of 50V for 20 minutes, 120V for 20 minutes, and a final 200V for 40 minutes. Proteins were transferred to an Immobilon-FL PVDF membrane (Millipore, IPFL07810) by semi-dry western blotting in a Trans-Blot® SD semi-dry transfer cell (Bio-Rad). The PVDF membrane and protein gel were sandwiched between two extra-thick blotting papers (Bio-Rad, 1703968) soaked in western blot transfer buffer (Tris-base 5.82mg/ml, glycine 2.93mg/ml, with 20% methanol v/v in dH₂O). Proteins were transferred to PVDF membrane at 0.06A for one, or 0.12A for two gels, for 1 hour. The PVDF membrane was blocked in Licor blocking buffer (Licor Biosciences, 927-40000) for 30 minutes with constant rocking at room temperature. The membrane was then incubated with primary antibody: (ROS-BC6 0.43µg/ml, or 3F4 0.1µg/ml) in 5ml Licor blocking buffer containing 0.1% v/v Tween in a 50ml Falcon tube, on a roller at 4°C overnight. The membrane was washed four times at approximately 15 minute intervals in PBST (PBS with 0.1% Tween 20). The membrane was incubated in 1µl goat anti mouse IRdye 680RD (Licor Biosciences, 926-68070) diluted in 5ml licor blocking buffer, 5µl tween, for 45 minutes, rolling at room temperature, and protected from light. The membrane was washed in PBST four times at 15 minute intervals, whilst protected from light. Proteins were visualised using the 700nm channel of a LICOR Odyssey and analysed using ImageStudio Software.

3.2.7 Immunofluorescence microscopy

To enable immunofluorescent staining cells were cultured on 12mm glass coverslips (VWR, 631-1577) placed in the bottom of 6 well plates. For analysis, coverslips were removed and placed into 12 well plates, and washed twice in PBS. Cells were fixed in 10% neutral buffered formalin for 30 minutes at room temperature. Cells were washed twice in PBS, permeabilised in 0.5% TritonX-100 for 10 minutes at 4°C, and washed twice in PBS. Cells were blocked with 5% goat serum in PBS for 1 hour prior to incubation with primary antibody (ROS-BC6 3.25µg/ml or 3F4 1.25µg/ml) for 1 hour at room temperature. Cells were washed twice in PBS before incubation with Goat anti-Mouse IgG (H+L) Highly

Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, A21236) at 1:400 for 45 minutes, protected from light at room temperature. Both primary and secondary antibodies were diluted in 5% goat serum. Cells were washed twice in PBS, before incubation with Hoescht nuclear stain (1:10000) for 2-3 minutes. Cells were washed twice in PBS, then twice in water before mounting to SuperFrost Plus™ slides (Thermo Scientific™, J1800AMNZ) using ProLong™ Gold or ProLong™ Diamond Antifade Mountant (Invitrogen™, P36970). Slides were analysed using a Leica DMLB upright fluorescent microscope with a Zaber encoded excitation filter wheel, Lumencor SOLA source and Coolsnap MYO low light mono camera/quad pass filter. Hoescht nuclear stain was detected using the violet 405nm filter (100ms exposure) and the Alexa 647 using the Far Red 647nm filter (800ms exposure). Image capture and analysis was performed with image J and micro-manager plug-in.

3.2.8 Transfection of RK13 cells

3.2.8.1 Transient expression: pcDNA3.1+ vector

RK13 cells were thawed and split 1 or more times and allowed to reach >75% confluency before each transfection experiment. Unless otherwise stated, cells were cultured in 10cm dishes (Nunc™, Thermofisher) for transient transfection experiments. Two 1.5ml Eppendorf tubes were prepared, one containing 7.5µg of plasmid DNA, 500µl serum-free Opti-MEM and 20µl P300 reagent, and a second containing 20µl Lipofectamine 3000 reagent (Thermo Fisher) and 500µl serum-free Opti-MEM. The two reaction mixtures were combined and incubated for 5-15 minutes at room temperature. Media was removed from the 10cm dish, and replaced with 7ml fresh media, DNA complexes were added dropwise around the dish, and swirled to mix. Cells were cultured for a further 24 hours before collection (see 3.2.5.4).

3.2.8.2 Stable expression: pcDNA3.1+ vector

Transient transfections of RK13 cells were performed as described in 3.2.8.1, however reactions were scaled down for a 6-well cell culture plate. Duplicate 6

well plates were transfected with varying lipofectamine and DNA ratios (see Table 3-5) and incubated for 24 hours. At this time one plate was analysed as a transient transfection, whilst the second was changed to fresh media for a further 24 hours, before supplementation with 1000µg/ml G418 (geneticin) for selection. The concentration of G418 necessary to kill 100% of RK13 cells after 10 days was previously established via a kill-curve experiment. Media containing G418 was replaced every 3-4 days for 14 days, after which cells were analysed by western blot (3.2.6) and immunofluorescence (3.2.7).

3.2.8.3 Transient expression: pTRE/pTetON vector

RK13 cells were co-transfected with pTREtight2_cPRP_3F4_TPQ and pTet-ON vectors to ensure their functionality in cell culture. RK13 cells were cultured in a 6 well plate containing one 12mm coverslip. Transfection was undertaken when cells were ~80% confluent as described in section 3.2.8.1, and Table 3-6. 24 hours following transfection, 1µg/ml doxycycline was added to induce expression. 72 hours following transfection, cells were collected on coverslips for analysis by immunofluorescence (section 3.2.7). The remaining cells in the culture plate were collected for analysis by western blot, as described in section 3.2.5.4.

3.2.8.4 Stable expression: pTRE/pTetON vector

RK13 cells were co-transfected with pTREtight2_cPRP_3F4_TPQ, pTet-ON and linear puromycin resistance marker (Takara bio, 631626) in order to generate a stable cell line expressing cervid PrP^C. Transfected cells were cloned and sub-cloned in order to increase expression levels. After transfection or each cloning stage, cells were frozen (3.2.5.2), passaged for continued culture (3.2.5.3), and PrP expression levels were characterised by immunofluorescence (3.2.7) and western blot (3.2.6). In all experiments, cells for analysis were induced by addition of doxycycline to the culture media (1µg/ml) for 24-48h prior to collection.

Initially five transfection protocols were tested and are summarised in Table 3-6. GFP controls for three of these protocols were analysed at 24h post transfection on a Zeiss Axiovert 25 inverted fluorescent microscope, 200ms exposure. 24 hours after transfection, cells were trypsinised and transferred to a 10cm dish with media supplemented with 2µg/ml puromycin, and cultured for 7 days for selection. The resistant colonies in each 10cm dish were pooled for analysis.

To generate clones with higher expression levels, the pooled puromycin resistant colonies from the most successful protocol (GB) were used to create a 1 in 5 dilution series in 10cm plates. Cells were cultured in puromycin supplemented media for 7 days until individual cell colonies of between 10-100 cells were visualised by microscopy and transferred to a 24 well plate by gentle scraping/suction using sterile 200µl pipette tips. After a further 7 days culture, 12 of the generated clones were analysed.

In a further attempt to increase expression levels, the clone showing expression (Col12) was further subcloned by dilution series as described above. 24 individual cell subclones were analysed by IF, and the four strongest expressing clones (SC-5, 13, 21, 22) were further passaged for repeated IF and WB analysis, and freezing.

3.2.9 Protein misfolding cyclic amplification (PMCA)

3.2.9.1 Preparation of brain homogenates for use in PMCA

Infected and uninfected brain homogenates used as PMCA substrate or seed are shown in Table 3-3. Natural scrapie brain homogenates were prepared by Khalid Salamat (KS). CWD positive brain samples were donated by Andrew Gill (Roslin Institute, Edinburgh) and were white-tailed deer experimentally inoculated with North American CWD isolates [399]. Cervid BSE samples and normal deer controls were provided by Mark Dagleish (Moredun Research Institute, Edinburgh) from experimental transmission of BSE in red deer [400]. Cervid BSE and CWD samples were prepared in a derogated Containment Level

3 (CL3) laboratory under aseptic conditions to produce a 20% (w/v) homogenate in 1X PBS. Homogenisation was achieved using an Omni electric homogenizer with disposable probes (Omni, 32750), and then passage of homogenate through 25G needle. The 20% homogenate was then diluted to 10% (w/v) in 2x PMCA buffer (see below) for use in PMCA, or in PBS for use in NAPTA precipitation. Normal brain homogenate was prepared in a CL2 lab under aseptic conditions to produce a 10% (w/v) homogenate in PMCA buffer. Brain tissue was dissected from the medulla and brainstem, meninges and or large blood vessels removed, and washed in PBS three times to remove residual blood. Homogenates were prepared using a probe homogeniser (see above) and passed through a series of needles of decreasing diameter (18G, 23G and 25G). Homogenates were frozen at -80°C.

Table 3-3: Brain homogenates used as PrP^C substrate or PrP^{Sc} seed for PMCA experiments.

Homogenate	Species	Use	Origin	Genotype	ID	Ref
Natural scrapie	Sheep	PrP ^{Sc} seed	Roslin scrapie flock	VRQ/VRQ		
CWD (Elk)	WTD	PrP ^{Sc} seed	NADC, USDA, Iowa, USA ¹	95QQ, 96GS	677	[399]
CWD (Mule)	WTD	PrP ^{Sc} seed	NADC, USDA, Iowa, USA ¹	95QQ, 96GS	643	[399]
Cervid BSE	Red deer	PrP ^{Sc} seed	Moredun Research Institute	TPQ/TPQ	Deer ID 25 R#4279	[400]
Control homogenate	Red deer	PrP ^C substrate	Moredun Research Institute	TPQ/TPQ	Deer ID 32 R#5863	[401]
				TPE/TPE	Deer ID 39 R#5864	[401]
Tg338	Tg mouse	PrP ^C substrate	INRAE-ENV, Toulouse, France	Ovine VRQ		
TgshpXI	Tg mouse	PrP ^C substrate	INRAE-ENV, Toulouse, France	Ovine ARQ		

¹National Animal Disease Centre, United States Department of Agriculture, Iowa, USA.

3.2.9.2 NaPTA precipitation and western blotting of PMCA seeds

500µl 10% (w/v) brain homogenate was thawed and sonicated for 15 seconds. Benzonase was added at 50 units/ml, 10mM MgCl₂ added at a 1:10 dilution, and samples incubated for 30 minutes with constant agitation. All incubations were performed at 37°C. An equal (1:1) volume of 4% (w/v) sarkosyl/PBS was added, then incubated for a further 10 minutes. Proteinase K solution was added to the sample to a final concentration of 50µg/ml, and incubated for 1 hour 15 minutes. To stop reaction, 11µl PMSF was added and the sample was vortexed at 1 minute intervals for 5 minutes. Pre-warmed (37°C) 4% w/v Sodium phosphotungstate octadecahydrate (NaPTA (Sigma, P6395)) containing 170mM MgCl₂, pH 7.4, was added to a final concentration of 0.3% (w/v) NaPTA, and samples incubated overnight with constant agitation. Samples were centrifuged at 15800 x g for 30 minutes and the supernatant discarded. Pelleted material was washed with 200µl of 0.1% (w/v) sarcosyl/PBS and 200µl of 250mM EDTA, vortexed to mix, and centrifuged again. To resuspend the pellet, 100µl of 0.1% sarcosyl/PBS was added, then the sample was heated to 100°C for 5 minutes on a heating block and sonicated briefly. The samples were then analysed by western blot as described in section 3.2.6.

3.2.9.3 Protein misfolding cyclic amplification (PMCA)

Experimental plans and substrate preparation by Amy Robinson, PMCA experiments and western blotting performed by Khalid Salamat (Roslin Institute).

A previously published microplate-based miniaturised bead PMCA protocol was used [402, 403]. Cell lysates or transgenic mouse brains were used to provide the PrP^C substrate, either TgshpXI (sheep ARQ allele) or tg338 (sheep VRQ allele), provided by Dr Olivier Andreoletti, (INRAE-ENV, Toulouse, France). Cell lysates were prepared as described in section 3.2.5.4. Mouse brains were homogenised in PMCA buffer (PBS, pH 7.2, 0.25% v/v Triton X100, 150mM NaCl, cOmplete Protease Inhibitor (Roche) at 4°C) using Dounce glass

homogenisers to create 10% (w/v) homogenate. Infected brain homogenates used as “seeds” were prepared as described in 3.2.9.1. PMCA reactions were performed in 96 well microtitre plates (Axgen®), with 45µl substrate and one Teflon bead (2.381 mm, Marteau & Lemarie, France) per well, and 5µl seed per well. Positive controls consisted of 10-fold serial dilutions of pooled brain homogenate from five BSE positive sheep, which had previously been titrated by end-point dilution in gene-targeted transgenic mice expressing sheep ARQ with an estimated titre of 8.5×10^7 ID₅₀/g. Negative controls included reactions seeded with brain homogenate from mock-infected sheep, or substrate-only (homogenate and/or lysate) controls. Microplates were sealed with 8-strip domed Axgen PCR tube caps and Parafilm. Plates were placed in a microplate horn attached to a programmable Misonix Q700 sonicator (QSonica). Distilled water maintained at 37°C was continually recirculated through the microplate horn throughout the experiment, using a peristaltic pump and waterbath. PMCA settings for a single round of amplification are described in (Table 3-4). Sonication amplitude was maintained between 50-65%. To perform another round of amplification, 5µl of product from each well was moved to 45µl of fresh substrate, and the PMCA repeated.

Table 3-4: Comparison of PMCA protocols tested for the optimisation of PMCA using cell lysate.

PMCA protocol	Number of cycles	Cycle sonication and amplification	Total round time (hours)	Ref
Protocol 1	144	5 second sonication 10 minute incubation	24	[395]
Protocol 2	96	10 second sonication 14 minute 50 second incubation	24	[402, 403]
Protocol 3	96	20 second sonication 29 minute 40 second incubation	48	[282]
Protocol 2 (modified)	192	10 second sonication 14 minute 50 second incubation	48	

3.2.9.4 *Western blotting of PMCA products*

To perform blotting of un-amplified reactions, 72µl of substrate was combined with 8µl of seed, and 18µl of this removed prior to amplification. After amplification, 18µl of the reaction product was removed, digested with 0.1mg/ml proteinase-K (Qiagen) for 1 hour at 37°C. An equivalent of 3.6µl of each PK-digested PMCA product was loaded onto NuPAGE™ 4 to 12% Bis-Tris (Life Technologies) gels using standard protocols as previously described (3.2.6), with the following changes: proteins were transferred onto nitrocellulose membrane (0.2µm, BioRad, 162-0112), blocking was performed in 5% (w/v) dried-skimmed milk in PBS, primary antibody ROS-BC6 was used at a final concentration of 0.25µg/ml, secondary antibody was Goat anti-mouse IgG Peroxidase conjugated (Thermo Scientific, 31431), and blot development and visualisation was performed using Clarity Western ECL Substrate (Bio-Rad) and Amersham Hyperfilm ECL. Molecular weight markers used were Precision Plus Protein Prestained Standards (Bio-Rad).

Table 3-5: Transient and stable transfection conditions attempted with pcDNA3.1+_TPQ_3F4 vector in RK13 cells.

Transfection	Cell state	Culture size	Plasmid preparation	Lipofectamine/Optimem	DNA/P300/Optimem	Reference
Transient	Monolayer, >75% confluency	10cm dish	-	20µl/500µl	7.5µg/20µl/500µl	Kurt <i>et al</i> 2017, Patricia Aguilar, pers. comm.
Transient	Monolayer, >75% confluency	10cm dish	-	20µl/500µl	5µg/20µl/500µl	
Transient	Suspension	10cm dish	-	20µl/500µl	7.5µg/20µl/500	
Transient	Suspension	10cm dish	-	20µl/500µl	15µg/20µl/500	
Transient and stable	Monolayer, >75% confluency	6 well plate	-	3.3µl/83µl	1.25µg/3.3µl/83µl	(Kurt <i>et al</i> 2017 scaled down)
Transient and stable	Monolayer, >75% confluency	6 well plate	-	3.75µl/125µl	2.5µg/5µl/125µl	Thermofisher protocol ¹
Transient and stable	Monolayer, >75% confluency	6 well plate	-	7.5µl/125µl	2.5µg/5µl/125µl	Thermofisher protocol ¹
Transient and stable	Monolayer, >75% confluency	6 well plate	-	3.75µl/125µl	5µg/5µl/125µl	
Transient and stable	Monolayer, >75% confluency	6 well plate	-	3.75µl/125µl	10µg/5µl/125µl	
Transient and stable	Suspension	6 well plate	-	3.75µl/125µl	2.5µg/5µl/125µl	
Transient and stable	Monolayer, >75% confluency	6 well plate	Linearised	3.75µl/125µl	2.5µg/5µl/125µl	
Transient and stable	Suspension	6 well plate	Linearised	3.75µl/125µl	2.5µg/5µl/125µl	

¹ https://tools.thermofisher.com/content/sfs/manuals/lipofectamine3000_protocol.pdf

Table 3-6: Transient and stable transfection conditions attempted with pTREtight2_cPRP_TPQ_3F4 and pTet-ON vectors in RK13 cells.

Transfection	Cell state	Culture size	Plasmid preparation	Lipofectamine/Optimem	DNA/P300/Optimem	Reference
Transient	Monolayer >75% confluency	6 well	-	3.75µl/125µl	0.8µg pTREtight2_cPrP_3F4_TPQ 1.6µg pTetON	
Stable* “GB”	Monolayer, 100% confluent	12 well	-	1.5µl/50µl	1µg pTREtight2_cPrP_3F4_TPQ 1µg pTetON 100ng puromycin marker	[385]
Stable* “MM”	Monolayer, 80% confluent	12 well	-	1.5µl/50µl	294ng pTREtight2_cPrP_3F4_TPQ 594µg pTetON 100ng puromycin marker	
Stable “MM-L”	Monolayer, 80% confluent	12 well	Linearised	1.5µl/50µl	294ng pTREtight2_cPrP_3F4_TPQ 594µg pTetON 100ng puromycin marker	
Stable “MM2x”	Monolayer, 80% confluent	12 well	-	1.5µl/50µl	588ng pTREtight2_cPrP_3F4_TPQ 1188µg pTetON 100ng puromycin marker	
Stable* “MM0.5x”	Monolayer, 80% confluent	12 well	-	0.75µl/50µl	147ng pTREtight2_cPrP_3F4_TPQ 297µg pTetON 50ng puromycin marker	

*GFP control vector also transfected using these conditions.

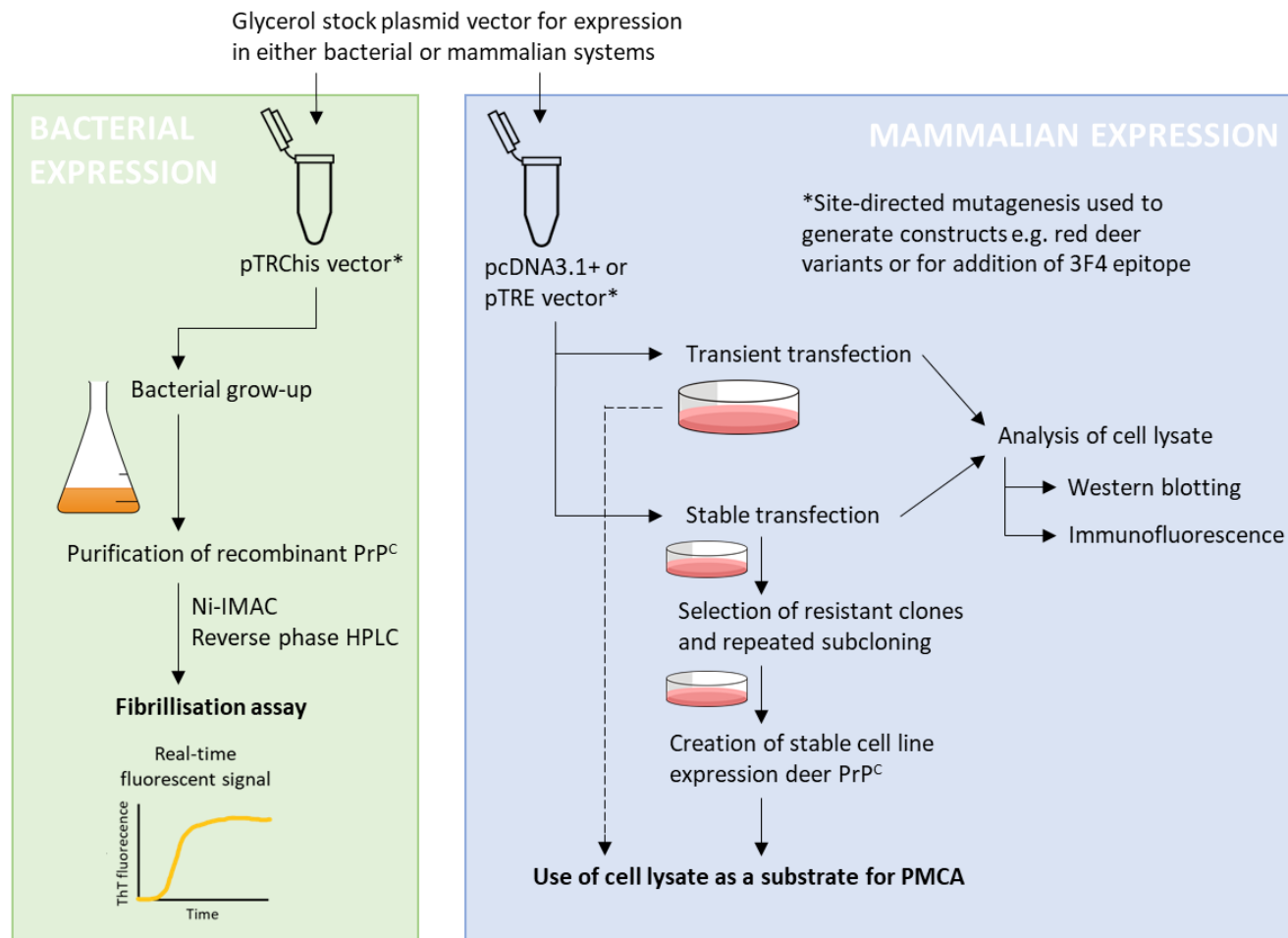


Figure 3-1: Schematic representation of approaches to produce recombinant PrP^C for use in in-vitro conversion assays

3.3 Results

3.3.1 Production of recombinant cervid prion protein and fibrillisation assay

The production of recombinant cervid prion protein was achieved via expression in *E. coli*, and purification of bacterial lysates by immobilised metal affinity chromatography (IMAC), desalting, refolding and reverse phase high-performance liquid chromatography (HPLC). Purified recombinant proteins were used in a fibrillisation assay which is a plate-based assay using shaking and ThT fluorescence to measure the propensity of recombinant proteins to spontaneously form fibrils, as described previously [379]. Initial experiments involved the purification of cervid PrP sequences known to be of differing susceptibilities in North American deer populations: elk 132L (GLSE) and 132M (GMSE), and white-tailed deer 96G (QGAS) and 96S (QSAS) (see 3.2.1.1). These were used as controls and as a proof-of-concept, which British cervid *PRNP* variants could be compared against. Purification of recombinant prion protein was successful, and initial fibrillisation assays showed the formation of amyloid and increase in ThT signal. However, this method of protein production yielded very small amounts of recombinant protein, usually only enough for a single

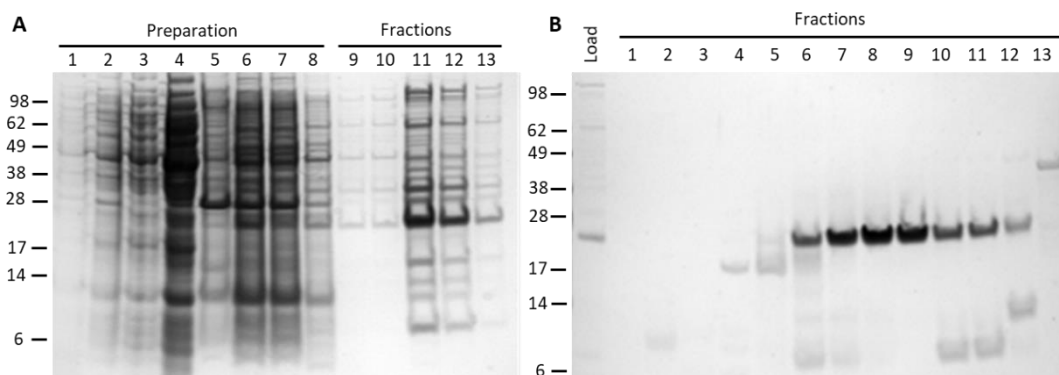


Figure 3-2: Purification of cervid prion protein (QGAS) by (A) NiIMAC and (B) reverse phase HPLC.

Protein purification is shown in stages following SDS-PAGE and staining with Instantblue™. (A) (1) Bacterial broth pre-IPTG induction (2) Bacterial broth post-IPTG induction (3) Discarded supernatant (4) Bacterial lysis product (5) Insoluble pelleted material (6) Soluble supernatant loaded for NiIMAC (7) Loading run-through (8) Column wash (9, 10, 11, 12, 13) Fractions 1-5. (B) Well 1 shows the loaded protein (fractions 3 and 4, desalted). Fractions 1-13 were collected following HPLC.

fibrillisation experiment. In a preliminary fibrillisation experiment, comparison of the two elk sequences showed a slight difference in lag times (Figure 3-3) when comparing fractions with the greatest purity from each batch. However, non-specific bands likely associated with protein degradation were often identified in fractions produced by HPLC (Figure 3-2b), and the kinetics of fibrillisation varied significantly between the different purified fractions (results not shown) making results difficult to interpret. Although attempts were made to improve the recombinant protein yield and purity, this was not successful, and due to the departure of one of my supervisors (Andrew Gill) with expertise in this technology, it was decided to shift focus to expression of cervid PrP in cell culture for use as substrate in PMCA assays.

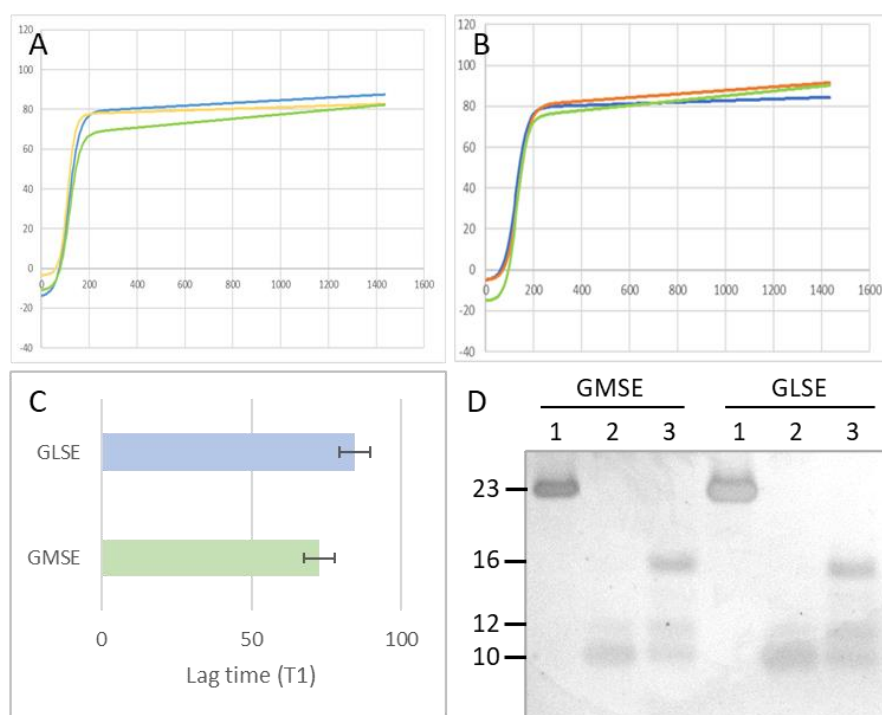


Figure 3-3: Proof of concept and initial results of fibrillisation kinetics for two variants (a) GMSE (Elk 132M) and (b) GLSE (Elk 132L).

(A, B) Kinetics are shown as ThT fluorescence (y axis) against time in minutes (x axis). Colours signify individual replicates of each recPrP. (C) Mean lag times and standard deviations were calculated for each fibrillisation replicate in one experiment (n=3) for GLSE and GMSE. (D) Confirmation of the presence of fibrils after PK digestion: (1) - PK, (2) +PK (3) Maturation at 80 °C, +PK. Bands of between 8 and 12kDa in the PK digested sample are consistent with the presence of amyloid fibrils, which are confirmed by the appearance of a 16kDa band after maturation.

3.3.2 Expression of cervid PrP in cell culture

I aimed to produce a cell line with high levels of expression of cervid PrP^C primarily for use as a substrate in PMCA assays. Rabbit kidney epithelial cells (RK13) were chosen due to their robustness in cell culture, lack of endogenous PrP expression, and well documented expression of various PrP^C sequences and use in cell infection studies in the literature (see introduction). Initially, I aimed to reproduce transient transfection using protocols described by Kurt *et al* [395], as this would allow for rapid generation of cervid PrP sequence variants via mutagenesis. First, mutagenesis was used to incorporate the 3F4 epitope into the consensus cervid *PRNP* sequence. This would allow specific detection of the PrP^{Sc} generated through *in-vitro* conversion of cell lysate, and differentiation from the PrP^{Sc} seed as the 3F4 monoclonal antibody binds cervid PrP with much lower affinity. Then each *PRNP* polymorphism would be introduced into the construct via mutagenesis and expressed in RK13 cells. Using cell lysate as a substrate in PMCA, the different cervid *PRNP* sequence variants expressed could be compared in terms of conversion efficiency against the baseline wild-type (TPQ) sequence.

3.3.3 Mutagenesis for the 3F4 epitope in cervid PRNP

The monoclonal antibody 3F4 is one of the most widely used tools in prion research. Introduction of the minimum epitope required for binding, which consists of a hepta-peptide between *PRNP* residues 109 and 115 [404], allows specific binding of the 3F4 antibody to the prion protein. The 3F4 epitope is encoded in the hamster and human variants of the prion protein, and the antibody binds strongly in these species due to a specific requirement for lysine at 109, and methionine at positions 112 and 115 (ruminant numbering), (Table 3-7). Other species which lack either one (sheep, cattle), or both (rat, mouse) methionine residues, are not recognised by the 3F4 antibody. Like sheep and cattle, deer PrP^C encodes only one methionine residue at 112, and valine at 115.

The pcDNA3.1+_TPQ vector encodes the full-length open reading frame of the red deer TPQ haplotype prion protein gene. Site-directed mutagenesis was used to change the amino acid at codon 115 from valine to methionine, to introduce the 3F4 epitope (pcDNA3.1_TPQ_3F4). Successful mutagenesis products were transformed into competent cells and DNA prepared for Sanger sequencing to confirm presence of the 3F4 epitope (Figure 3-4).

Table 3-7: Species comparison of the 3F4 epitope.

Codon numbering shown is standard for ruminant species. *Codon 112 varies depending on mouse species.

Species	109	110	111	112	113	114	115
Human	K	T	N	M	K	H	M
Hamster	K	T	N	M	K	H	M
Mouse	K	T	N	M/L/F*	K	H	V
Sheep	K	T	N	M	K	H	V
Deer	K	T	N	M	K	H	V
Deer + 3F4	K	T	N	M	K	H	M

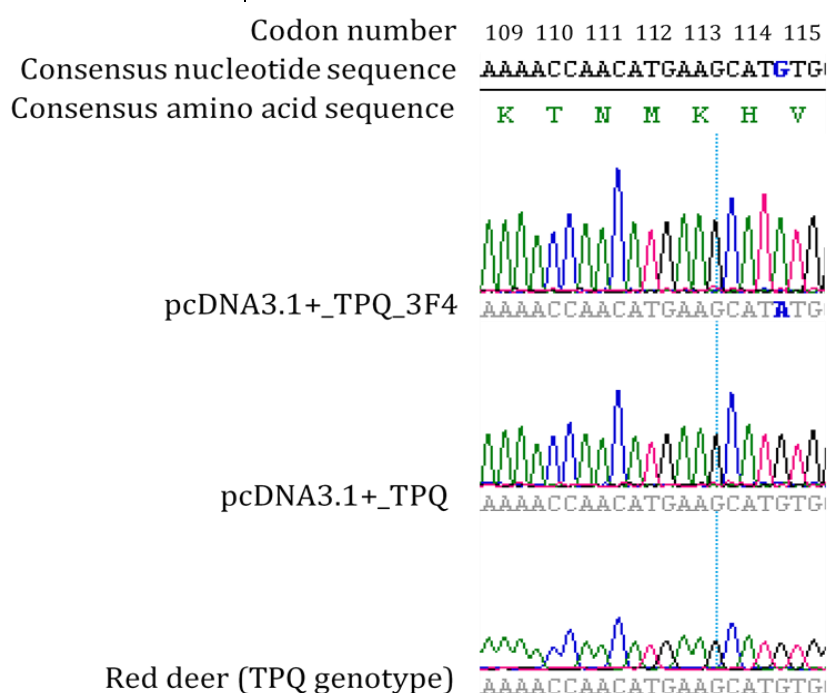


Figure 3-4: Sequencing chromatograms to show mutagenesis for the 3F4 epitope in pcDNA3.1+ vector with cervid TPQ PRNP gene

3.3.4 Transient transfection of RK13 cells with pcDNA3.1+_TPQ_3F4

To induce the expression of the cervid prion protein in cell culture, we transiently transfected rabbit kidney epithelial cells (RK13) with the pcDNA3.1+_TPQ_3F4 vector described above. A previously generated RK13 expressing stable cell line, Rov9 [385], expresses the ovine VRQ *PRNP* sequence (Val-136, Arg-154 and Gln-171) under the control of a tetracycline-inducible promoter. Rov9 is included throughout this chapter as a positive control for cell expressed PrP^C.

Figure 3-5 shows the results of a transient transfection of RK13 cells with pcDNA3.1+_TPQ and pcDNA3.1+_TPQ_3F4 vectors. Duplicate western blots were performed using ROS-BC6 and 3F4 antibodies. The human prion protein (as demonstrated by recombinant PrP) encodes the 3F4 epitope and therefore

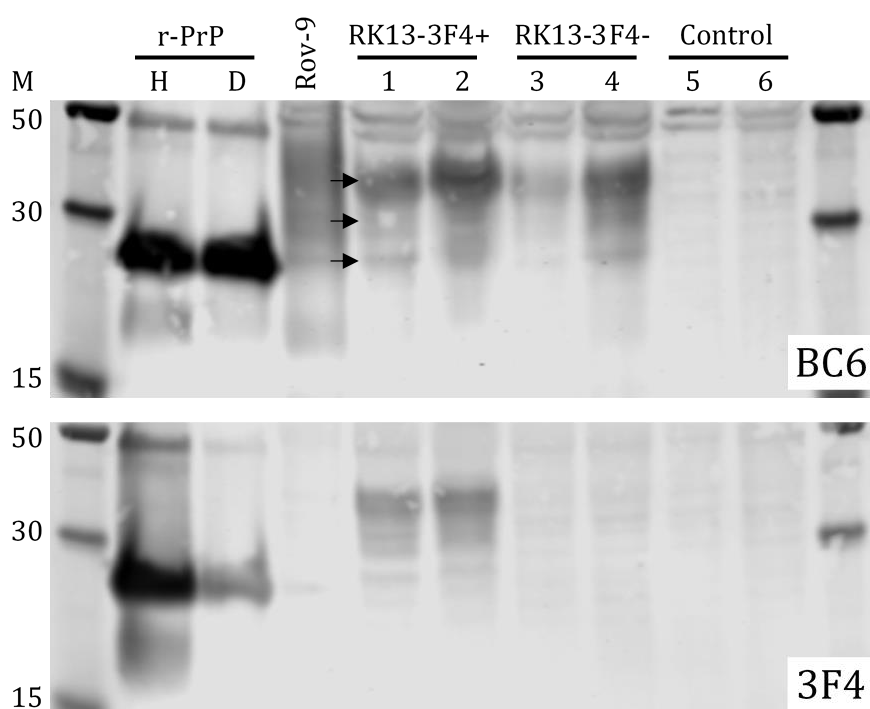


Figure 3-5: Expression of cervid PrP^C in RK13 cells after transient transfection with the pcDNA3.1+_TPQ_3F4 vector.

Recombinant human and deer PrP (400ng) are shown in lanes 1 and 2. RK13 cells transfected to express deer PrP with (3F4+) and without (3F4-) epitope are shown, transfected at 70% confluence (1,3) or 90% confluence (2,4). Control RK13 cells with (5) or without (6) lipofectamine treatment. Equal protein loading for RK13 cells, Rov9 loaded at ~50%.

is detected equally by the ROS-BC6 and 3F4 antibodies (Figure 3-5). Deer prion protein shows a significant reduction in signal when detected using the 3F4 antibody; residual signal from the deer PrP may be due to a low level of cross-specificity, or more likely to due to excess of protein loaded. The recombinant proteins are detected as a single band at approximately 25kDa due to the lack of post-translational modification of the protein. The typical three band PrP^C pattern at corresponding to the di-, mono- and un-glycosylated forms of the protein respectively, are present in the Rov9 and RK13 cells transfected at low (70%) and high (90%) confluence (see arrows). In RK13 cells transfected with deer *PRNP* encoding the 3F4 epitope (pcDNA3.1+_TPQ_3F4), PrP^C bands are seen in both the ROS-BC6 and 3F4 blots. RK13 cells transfected with deer *PRNP* lacking the 3F4 epitope (pcDNA3.1+_TPQ) are only detectable with the ROS-BC6 antibody. The RK13 control shows no detectable PrP^C expression in either blot. We can therefore conclude that 1) there is expression of cervid prion protein in RK13 cells and 2) the mutagenesis of cervid *PRNP* to introduce the 3F4 epitope allows for the expression and specific detection of PrP^C in cell culture lysates with the 3F4 antibody. Repeated attempts to express deer PrP in RK13 were variably successful, however generally expression levels were significantly lower than those seen in both the Rov9 cell line and in deer brain homogenates (Figure 3-6). Variations of the transfection protocol were therefore explored in the attempt to optimise transfection efficiencies and increase protein expression (summarised in Table 3-5). Low transfection efficiencies may be caused by DNA degradation or contamination of the DNA with endotoxins. Therefore, a specific kit to produce endotoxin-free transfection-grade plasmid DNA was used for plasmid preparation, and plasmids were used for transfection within 1-2 weeks of preparation. Varying DNA concentrations were attempted, to assess whether increased DNA concentration may improve transfection efficiencies. Likewise, incubation of DNA with the lipofectamine reagent was increased from 5 to 15 minutes to promote complex formation. Low passage cells (<25) were used in transfection experiments, at a range of confluence never less than 75%. A previously published protocol used by [395] was initially tested, as well as the manufacturer recommended protocol for lipofectamine

3000. Cells were transfected in monolayer, as well as in suspension (following trypsinisation) which is thought to increase the available surface area for interaction with lipofectamine:DNA complexes. Immunofluorescent detection of PrP expression allowed comparison of expression levels in Rov9 to transfected RK13 cells (Figure 3-7). Whilst reasonable transfection efficiencies could be achieved by transient transfection, as seen in the transfected RK13 cells and the GFP control, this was variable between experiments. Lipofectamine toxicity was also significant in all experiments, regardless of cell confluence, lipofectamine or DNA volume used. Lipofectamine toxicity can be seen in transfected cells in Figure 3-7, and was also present in GFP controls though some areas (pictured) were less affected. Detection of PrP expression was likewise variable by western blotting, and additional western blots were performed to ensure that PrP^C was not being degraded or lost during the lysate preparation stage (results not shown).

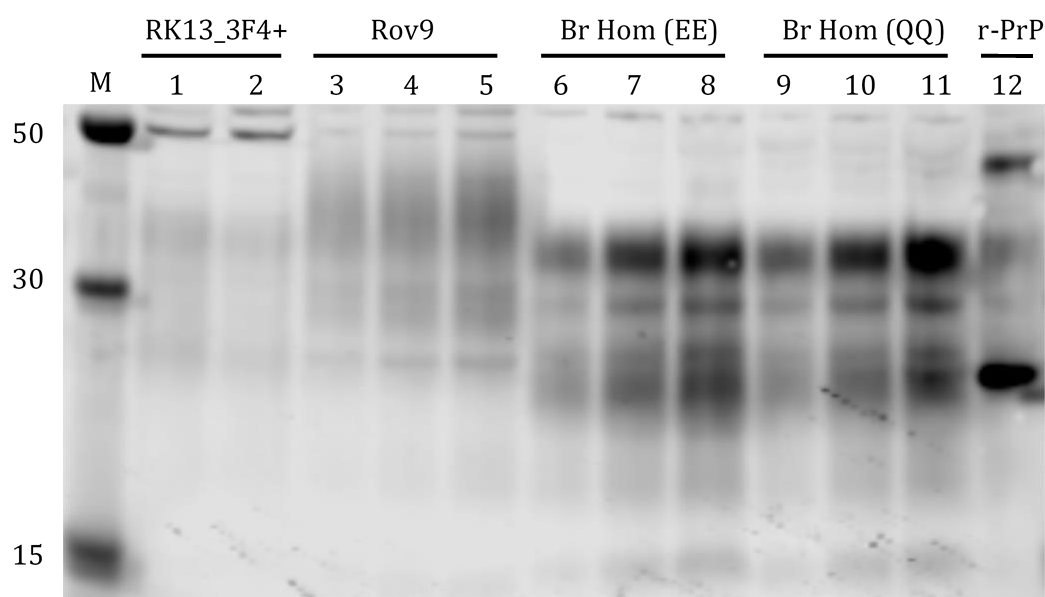


Figure 3-6: Comparison of PrP^C expression between cell lysates (RK13_3F4+ and Rov9) and red deer brain homogenates of 226EE and QQ genotypes.

Detection of PrP^C using ROS-BC6 antibody. Loading of brain homogenates and Rov9 as follows: 1ul (3,6,9), 2ul (4,7,10), 5ul (5,8,11) and 15ul (1,2). Recombinant deer PrP (200ng) is shown in (12).

In summary, despite various attempts to optimise the transient transfection of RK13 cells, only variable low-level expression of PrP^C was achieved. Whilst transient transfection is a faster method for the generation of cell lysate expressing many different PrP^C variants, the failure to produce a consistent and high level of expression would make it difficult to produce substrate needed for optimisation of a quantitative assay. I therefore decided to attempt to produce a stable cell line expressing deer PrP^C with the 3F4 epitope. Whilst the initial development of a stable cell line takes considerable time due to the necessary transfection, antibiotic selection and sub-cloning phases, the benefits of run-to-run consistency, selection for highly expressing clones, and the ability to easily grow large batches makes this a good choice for producing substrate for assay development.

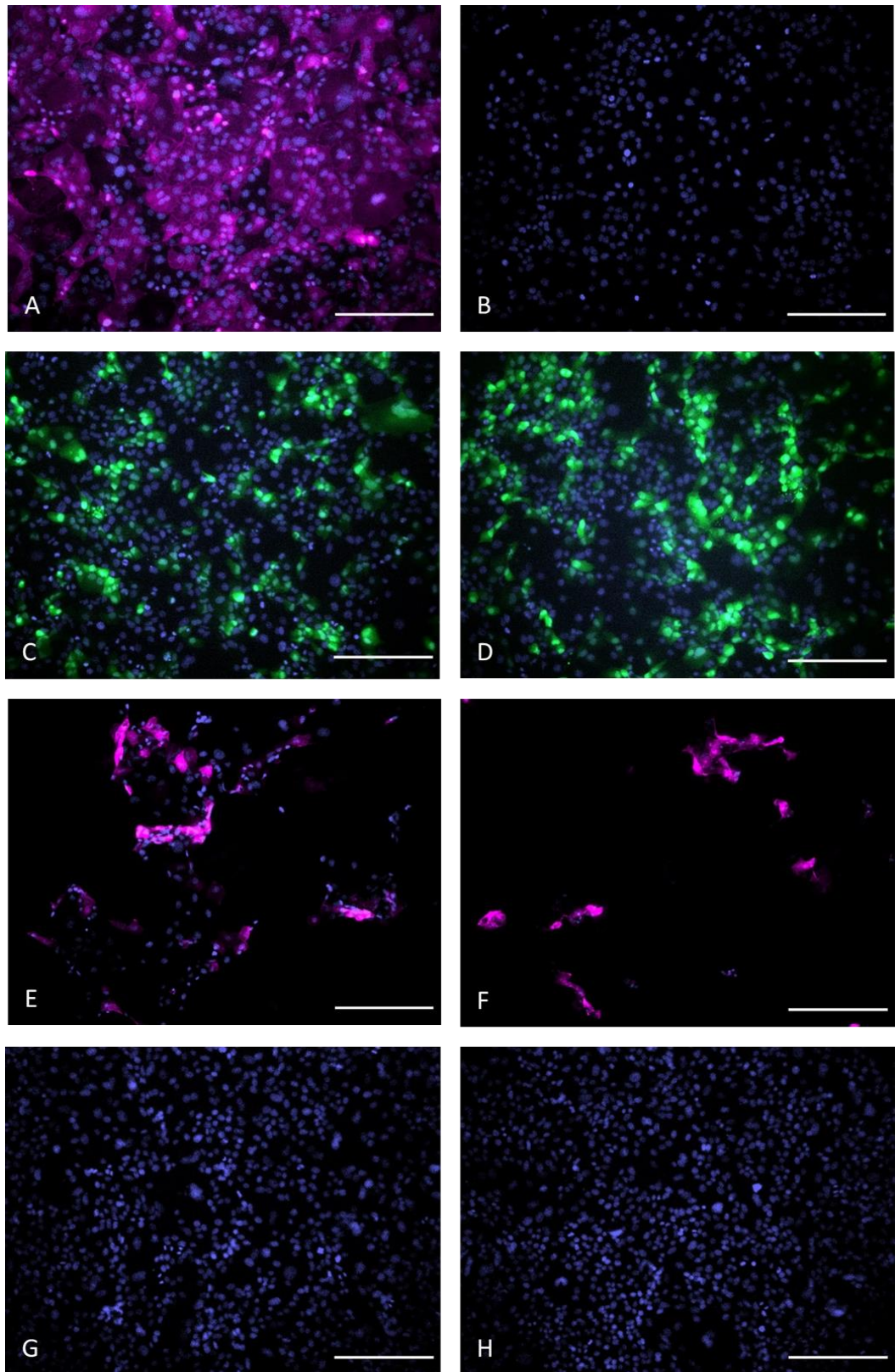


Figure 3-7: Immunofluorescence of PrP^C expression using ROS-BC6 in RK13 cells transiently transfected with pcDNA3.1+_TPQ_3F4.

A) Rov9 B) RK13 untransfected control C) RK13 expressing GFP, D) RK13 expressing GFP, transfected in suspension E) RK13 with pcDNA3.1+_TPQ_3F4 F) RK13 with pcDNA3.1+_TPQ_3F4, transfected in suspension G) Rov9 secondary antibody only control H)Rov9 unstained control. Scale bars: 200 μ m.

3.3.5 *Stable transfection of RK13 cells using pcDNA3.1+_TPQ_3F4*

The pcDNA3.1+ vector contains the neomycin resistance gene for selection of stable cell lines. A stable transfection of RK13 cells with the pcDNA3.1+_TPQ_3F4 vector was undertaken as described (3.2.8.2). RK13 cells were transfected with the pcDNA3.1+ vector, either circular or linearised, in either monolayer culture or suspension. Linearisation of the vector (by BglII digest) decreases the chance of an integration into the genome in a way that disrupts the gene of interest. An initial experiment was performed in duplicate, to allow direct comparison of transient transfection (Figure 3-7) with stable transfection under the same transfection conditions (Figure 3-8). For stable transfections, the vector was selected for in G418 antibiotic media, which produced 100% death of un-transfected cells.

The analysis of these stable transfections suggests that cells were surviving and expressing very low levels of protein in comparison to the Rov9 cell line. Occasional highly expressing cells were visualised upon immunofluorescence, (Figure 3-8, panel B). However, due to the infrequency of these cells, it was unlikely that further subcloning of this cell line would be successful in producing a subpopulation of stable highly expressing cells. One explanation for lack of high-level expression may be due to the nature of the expression vector. Whilst pcDNA3.1+ contains a CMV promoter for high level expression, it may be the case that constitutive expression led to unwanted effects or a lower level of protein expression when compared to the doxycycline inducible expression in Rov9 cells. I therefore decided to subclone the *PRNP* insert from the pcDNA3.1+ vector into the same expression system originally used to produce Rov9 cells, to determine if better levels of expression could be achieved.

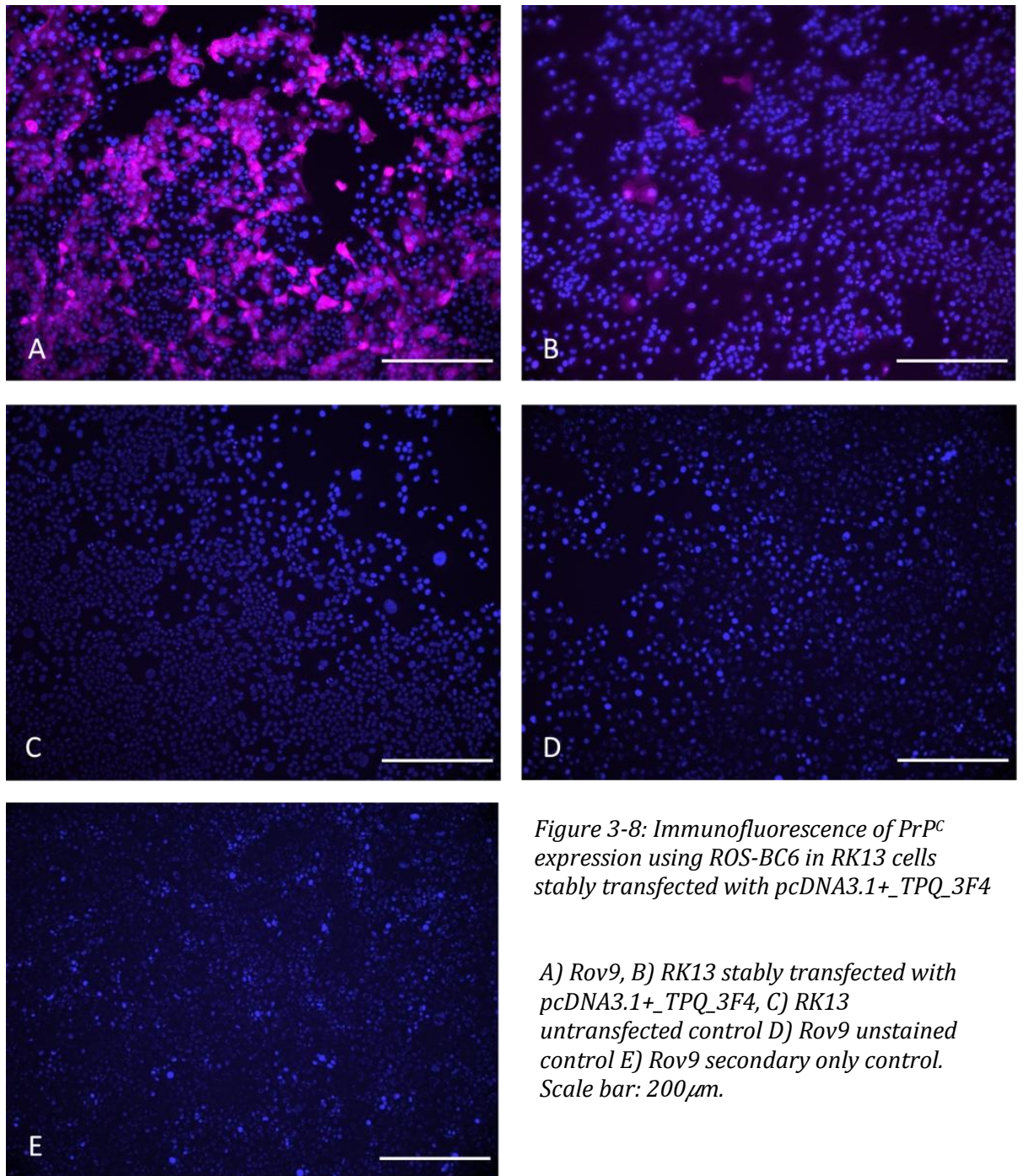


Figure 3-8: Immunofluorescence of PrP^C expression using ROS-BC6 in RK13 cells stably transfected with pcDNA3.1+_TPQ_3F4

A) Rov9, B) RK13 stably transfected with pcDNA3.1+_TPQ_3F4, C) RK13 untransfected control D) Rov9 unstained control E) Rov9 secondary only control. Scale bar: 200 μ m.

3.3.6 Construction of *PrP*-expressing vectors

3.3.6.1 Preparation of *pTRE* and *pTet-ON* plasmids

The Rov9 cell line was produced using *pTRE* and *pTet-ON* plasmids, in combination with a puromycin marker for selection of resistant clones. The Tet-ON inducible expression system allows for tightly regulated and high-level expression of the gene of interest when induced by doxycycline. *pTet-ON* and *pTRE* plasmids were obtained from external sources (3.2.1.2) and confirmed plasmid identity by performing multiple restriction enzyme digests (Panel A, Figure 3-9). A lane 1 shows the linearised *pTet-ON* plasmid (7.4kb), and lane 2 shows *pTet-ON* digested producing 6047bp and 1353bp fragments (latter not visible). Lanes 3 and 4 show the *pTREtight2* plasmid linearised at 2932bp, and digested to produce fragments at 2894bp and 38bp (latter not visible). The *pcDNA3.1+_TPQ_3F4* vector was digested to provide the cervid *PRNP* insert flanked by restriction sites *NheI* and *XbaI*, for use in subcloning (Panel B, Figure 3-9). Lane 1 shows the undigested vector in the supercoiled, linear and nicked states. Lane 2 shows the linearised plasmid, at 6103bp. Lane 3 shows the digested vector, with the *pcDNA3.1+* backbone at 5320bp, and 783bp *PRNP* insert (see arrowhead).

3.3.6.2 Subcloning of cervid *PRNP* into *pTREtight2*

The digested *pcDNA3.1+_TPQ_3F4* vector was separated by electrophoresis to allow excision of the *PRNP* insert and purification for use in subcloning. The *PRNP* insert was ligated into a linearised *pTREtight2* vector at restriction sites *NheI* and *XbaI*. Panel C, Figure 3-9 demonstrates the restriction enzyme digest of two successful subclones: the *BamHI* enzyme cuts at a restriction site on the *pTREtight2* backbone (2899bp) and is not present in the *PRNP* gene. The *NcoI* enzyme cuts a restriction site approximately 1/3 into the *PRNP* sequence. If the *PRNP* insert is present and correctly oriented, bands at 3448bp and 219bp are produced, as demonstrated in lanes 2 and 3 of (Panel C, Figure 3-9). To confirm

orientation of *PRNP* gene and presence of 3F4 epitope, new vectors were sequenced.

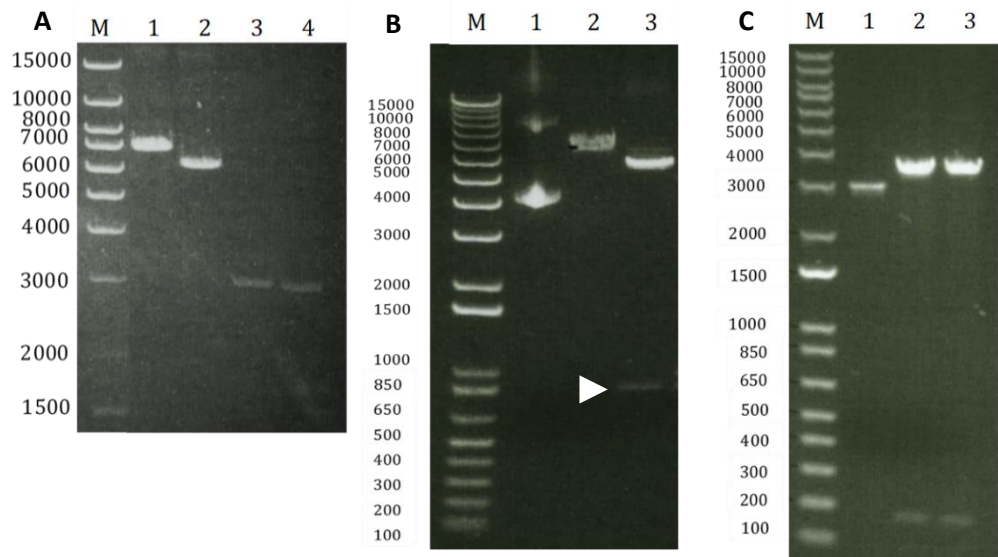


Figure 3-9: Restriction enzyme digests of pTet-on and pTRETight2 plasmids to confirm plasmid identity and subcloning of cervid PRNP.

(A) Restriction enzyme digest of pTet-on and pTRETight2 plasmids. Well: (1) pTet-on + XbaI (2) pTet-on + XbaI and NheI, (3) pTRETight2 + XbaI, (4) pTRETight2 + XbaI and NheI.

(B) Restriction enzyme digestion of pcDNA3.1+_TPQ_3F4. (1) Undigested, (2) BglII, (3) NheI and XbaI.

(C) Restriction enzyme digests of pTRETight2_cPrP_3F4_TPQ. (1) pTRETight2 empty vector + XbaI, (2) pTRETight2_cPrP_3F4_TPQ_1 + BamHI and NcoI, (3) pTRETight2_cPrP_3F4_TPQ_2 + BamHI and NcoI

3.3.7 Cervid PRNP expression in RK13 cells using pTRE and pTet-ON plasmids

A transient transfection of RK13 cells was performed with the pTREtight2_cPrP_3F4_TPQ and pTet-ON vectors to ensure functionality in cell culture. Expression of PrP^C was confirmed on both immunofluorescence and by western blotting (Figure 3-10).

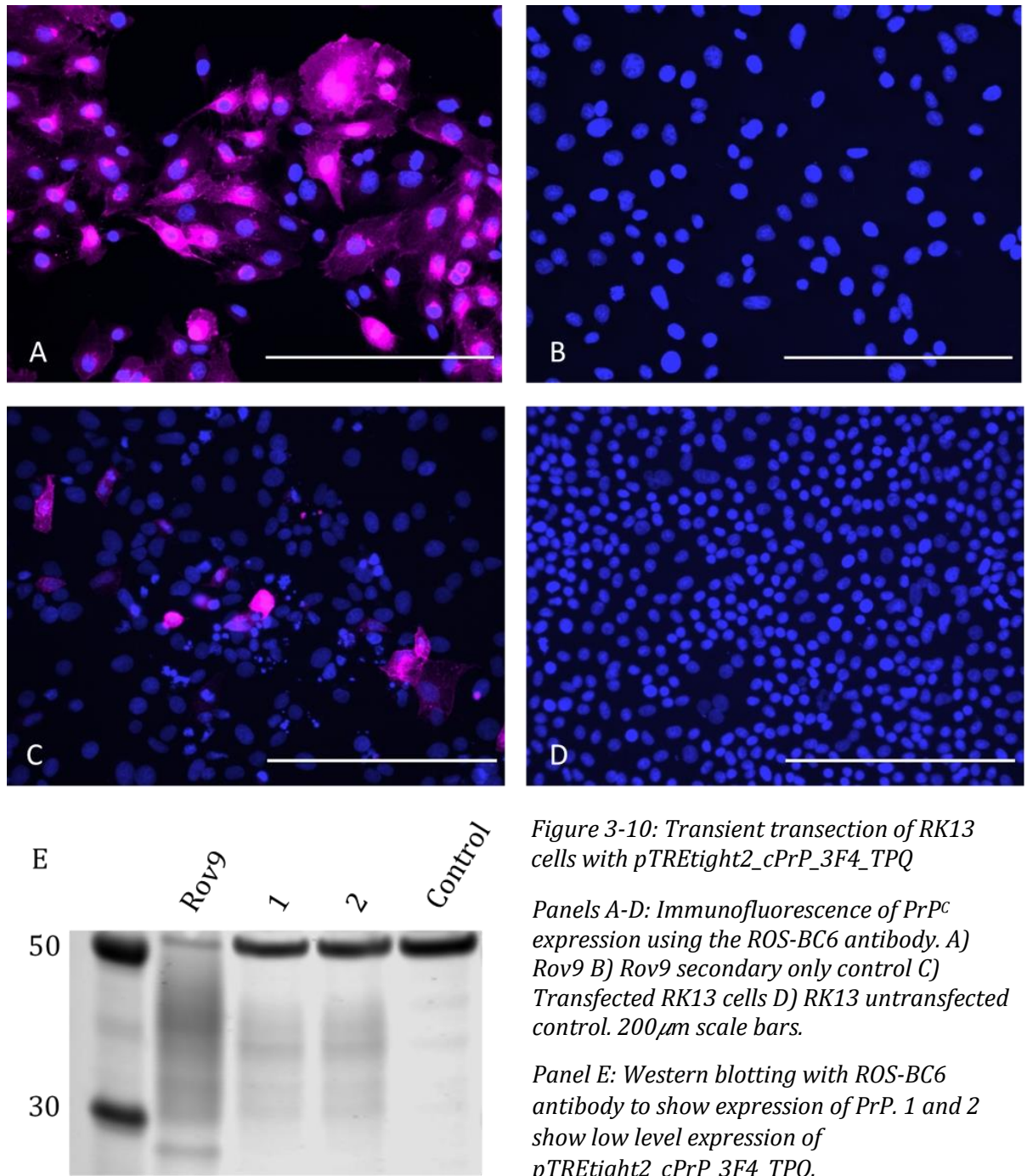


Figure 3-10: Transient transfection of RK13 cells with pTREtight2_cPrP_3F4_TPQ

Panels A-D: Immunofluorescence of PrP^C expression using the ROS-BC6 antibody. A) Rov9 B) Rov9 secondary only control C) Transduced RK13 cells D) RK13 untransfected control. 200 μ m scale bars.

Panel E: Western blotting with ROS-BC6 antibody to show expression of PrP. 1 and 2 show low level expression of pTREtight2_cPrP_3F4_TPQ.

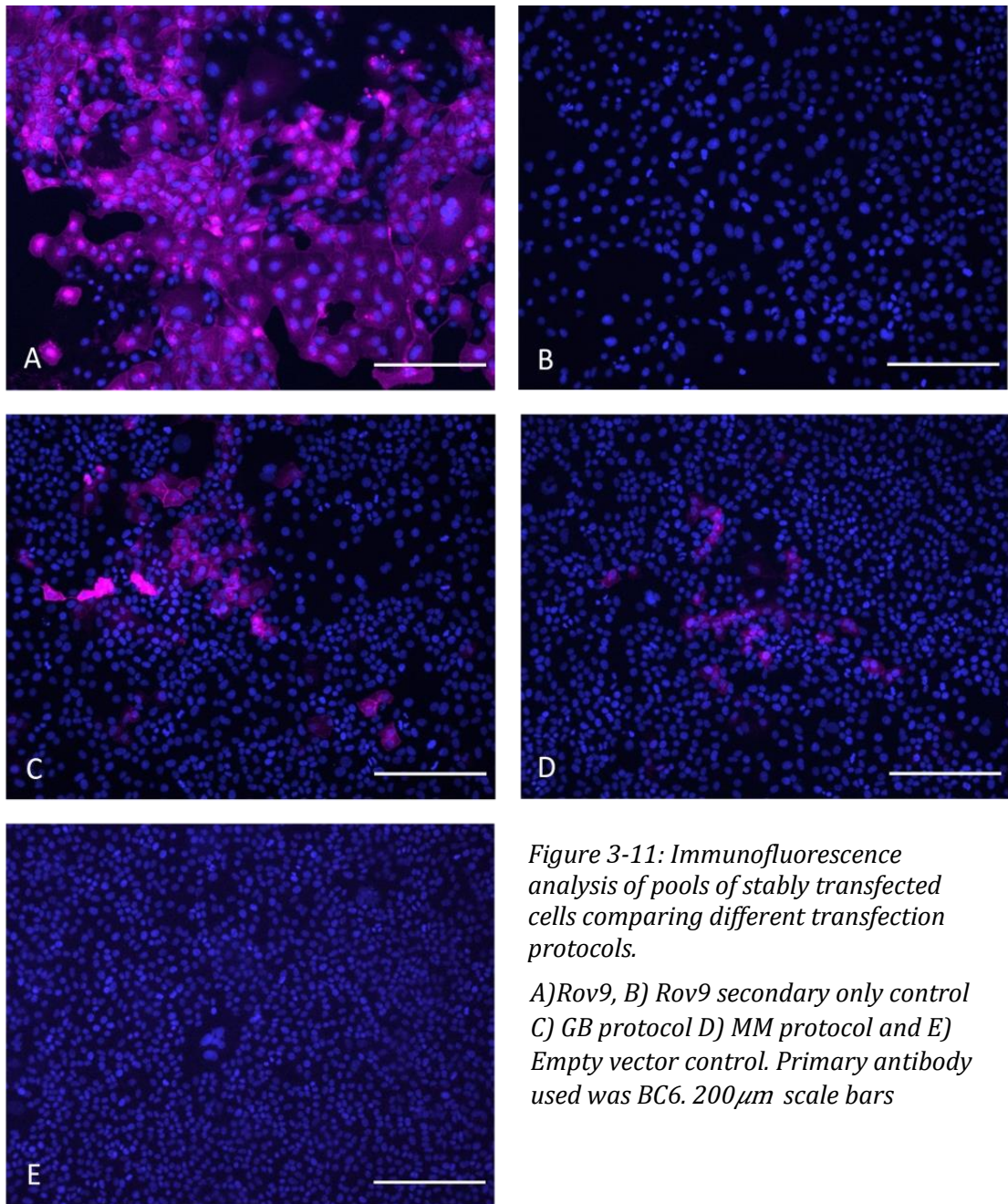
3.3.8 Stable transfection, cloning and characterisation of stably transfected RK13 cell clones

RK13 cells were transfected with the vectors described above, with the addition of a linear puromycin resistance marker for selection. Five protocols were tested to determine the best combination of cell confluency, vector concentration and ratio, and lipofectamine volume, and are summarised in Table 3-6. Three of the protocols (a published protocol “GB”, an adapted protocol “MM”, and a 50% protocol “MM0.5x”) were also used to transfect a GFP-expressing vector into RK13 cells, to allow comparison of these methods at 24 hours post-transfection by fluorescence microscopy. Transfection efficiencies were reasonable, at approximately 50%, and little difference was determined between the three methods at this point (results not shown). Controls including untransfected RK13 cells, and RK13 cells transfected with the pTRETight2_cPrP_3F4_TPQ and pTet-ON vectors without the puromycin resistance marker, died within a week of puromycin selection. Resistant colonies from each transfection protocol were pooled for analysis by immunofluorescence microscopy following induction of protein expression by addition of doxycycline. All tested protocols had cells expressing PrP^C detected by the ROS-BC6 antibody, which were scattered in clusters throughout the slide. Expressing cells were occasional in protocols “MM2x”, “MM0.5x” and “MM-L”, more frequent in protocol “MM”, and most frequent in protocol “GB” (Figure 3-11). No positive staining was seen in cells transfected with the empty pTRETight2 vector. Western blotting of lysates prepared from the cell pools failed to show any expression, suggesting a low level of expression present overall.

The most successful pool of transfected cells (protocol “GB”) was then used for cloning with the aim of selecting a clone with high levels of PrP expression. Only one of twelve clones (clone 12) was found to express detectable levels of PrP on immunofluorescence, as seen in Figure 3-12 panel C. The other 11 clones and

the empty vector control showed no positive staining. Clone 12 had a clustering of highly expressing cells on a background of lower expressing cells, seen more clearly in panel D. The highly expressing cells appear similar in expression level to Rov9. Unexpectedly, this expression was undetectable by western blotting, suggesting either a lower level of expression than seen by immunofluorescence or possibly degradation of the lysate prior to western blot.

Clone 12 was further sub-cloned, to attempt to select a clone with PrP expression at a level comparable with Rov9. 24 sub-clones were analysed by immunofluorescence, and were found to have variable levels of expression. 4 of the sub-clones with the highest PrP expression levels (SC5, SC13, SC21 and SC22) were selected for further passage and analysis. SC5 had the highest levels of expression upon immunofluorescence, (Figure 3-13 and Figure 3-14). Clusters of highly expressing cells were present in SC5 stained with both ROS-BC6 and 3F4 antibodies. Cells positive with 3F4 were less common than with ROS-BC6, however it was difficult to interpret these results due to the lack of available cell lines to use as a control for the 3F4 antibody in immunofluorescence. Whilst sub-cloning did increase the numbers of highly expressing cells when compared to the original clone-12, immunofluorescence still showed only clusters of cells expressing at levels comparable to Rov9, with the majority of cells expressing at much lower levels. Western blot analysis shows a similar level of expression for all four lysates, which was weaker in comparison to Rov9, but increased compared to Clone 12, (Figure 3-13; panel G). Permeabilised SC5 cells showed a similar pattern of staining to Rov9, with accumulation of PrP^C at the cell surface and in the golgi body using both ROS-BC6 and 3F4 antibodies (Figure 3-14). PNGase treatment of cell lysates to remove N-linked glycans produced an unglycosylated PrP^C band for Rov9 and SC5 lysates, but not RK13 control cells. A prominent band was also present at ~17kDa which is likely the C1 fragment of the prion protein.



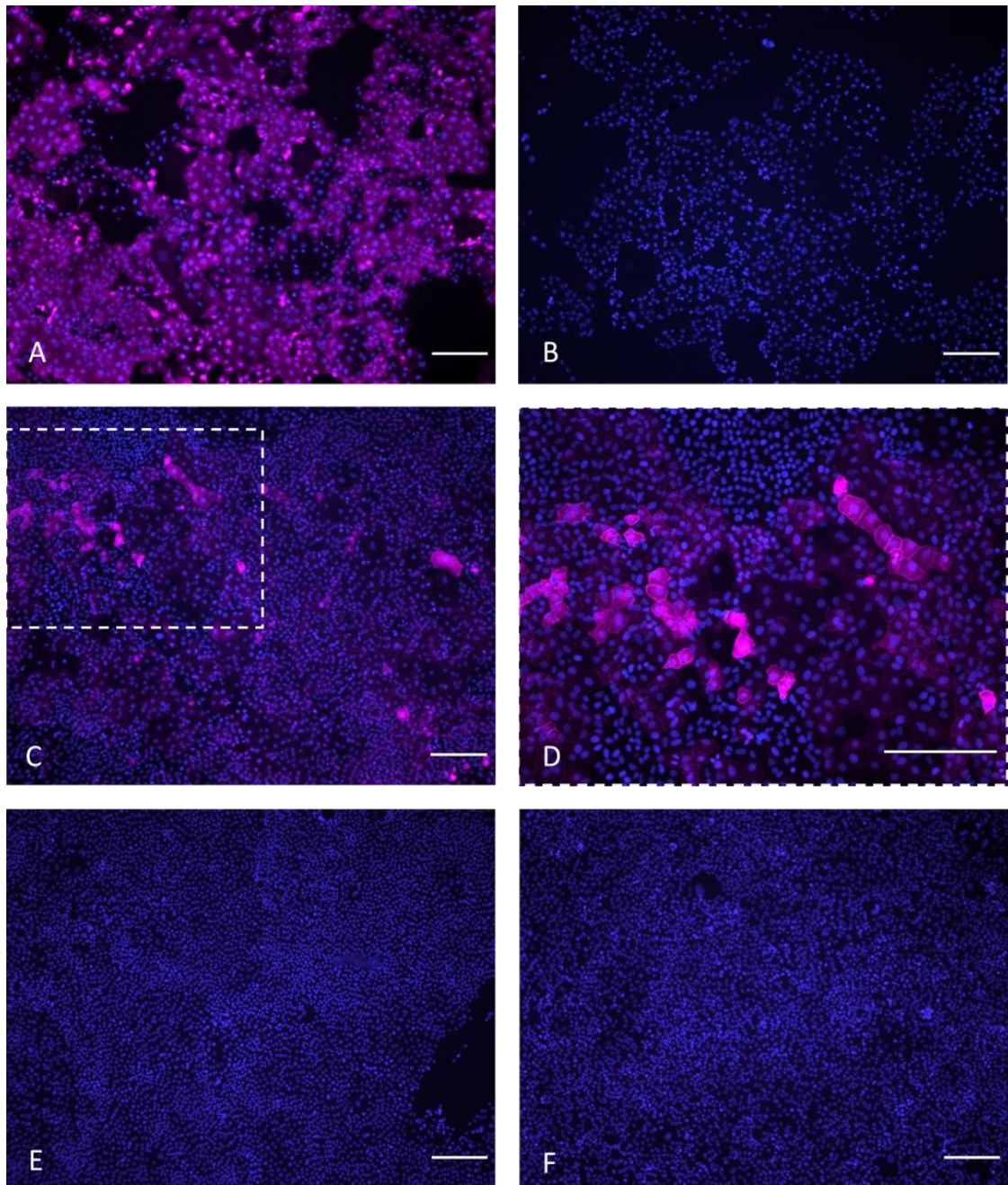


Figure 3-12: Subcloning of RK13 cells transfected with pTREtight2_cPrP_3F4_TPQ. Immunofluorescence using the ROS-BC6 antibody.

A) Rov9 B) Rov9 secondary only control C) Clone 12 D) Clone 12 at higher magnification E) Clone 1 F) Empty vector control. Scale bars: 200 μ m

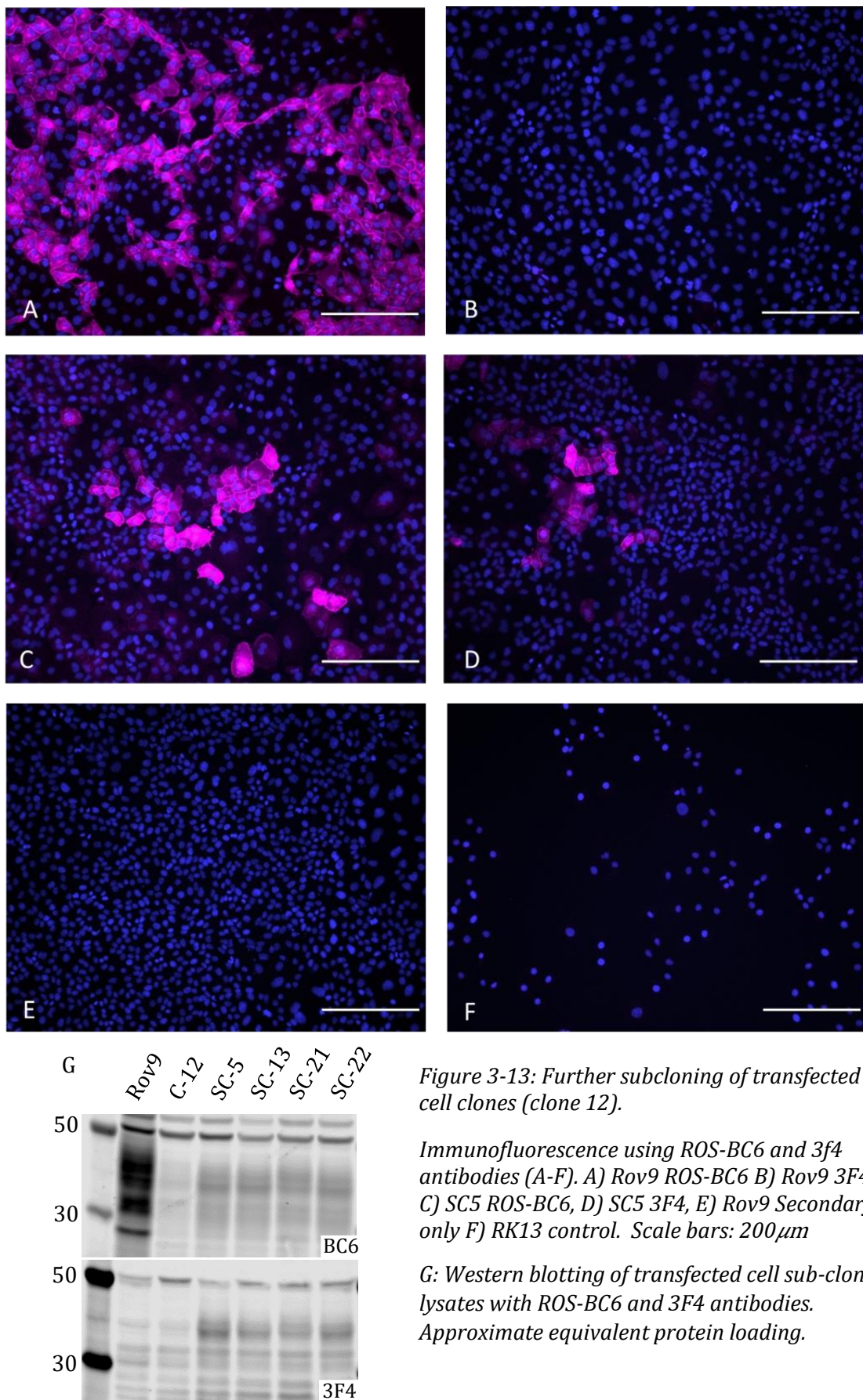
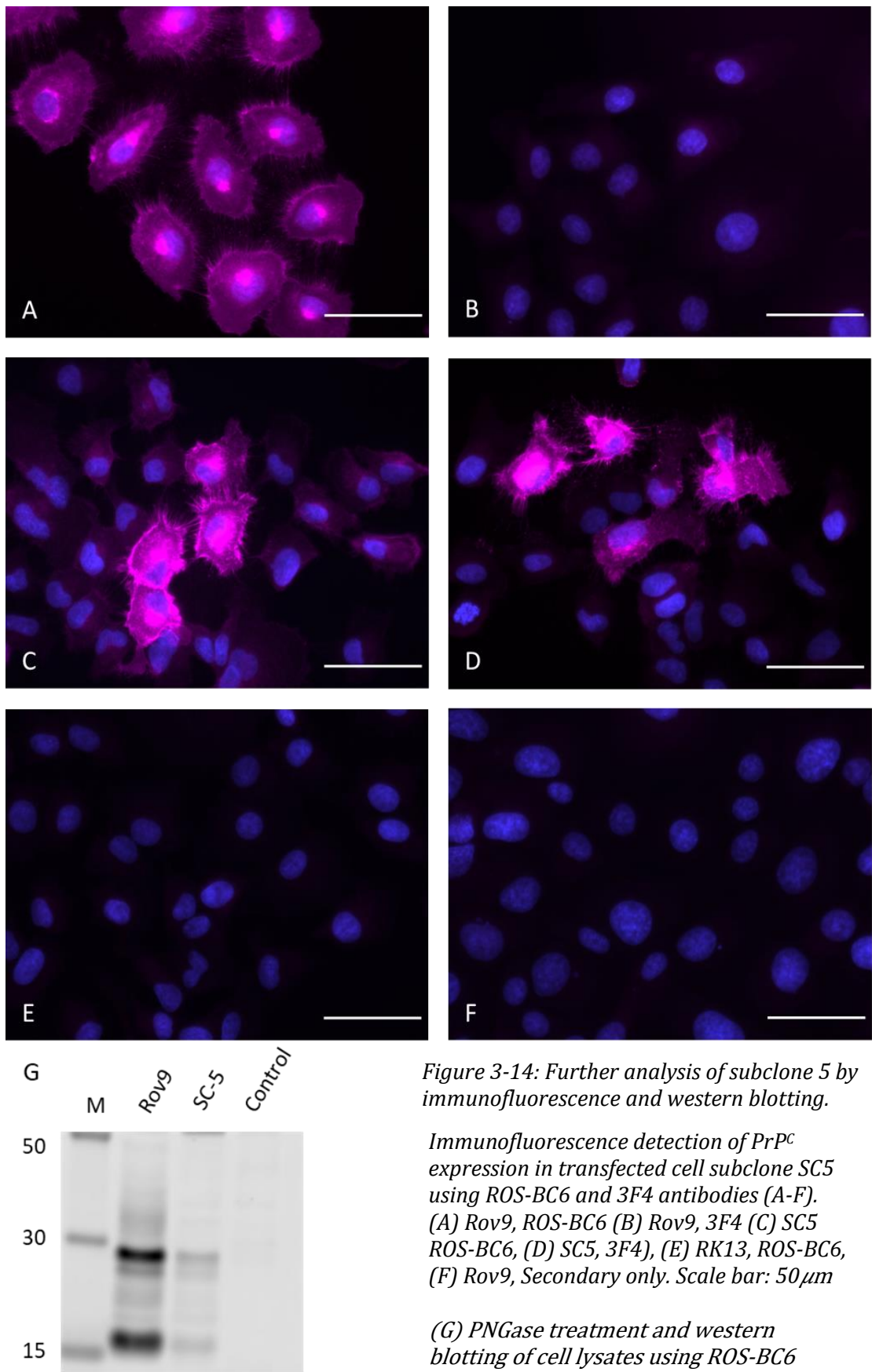


Figure 3-13: Further subcloning of transfected cell clones (clone 12).

Immunofluorescence using ROS-BC6 and 3f4 antibodies (A-F). A) Rov9 ROS-BC6 B) Rov9 3F4 C) SC5 ROS-BC6, D) SC5 3F4, E) Rov9 Secondary only F) RK13 control. Scale bars: 200 μ m

G: Western blotting of transfected cell sub-clone lysates with ROS-BC6 and 3F4 antibodies. Approximate equivalent protein loading.



3.3.9 Protein misfolding cyclic amplification (PMCA) using cell lysate as a substrate

3.3.9.1 Optimisation of PMCA using Rov9 lysate

PMCA is a widely used technique in prion research. The protocol for PMCA amplification can vary widely depending on lab, due to differences in reagents, equipment, sonication protocols and importantly, the seed/substrate combination to be amplified. We therefore began by optimisation of a cell-based PMCA method using ovine *PRNP* (Rov9) cell lysate using our lab equipment and protocols and compared this to other published protocols (Table 3-4) [282, 395, 402, 403]. Whilst our lab protocol (protocol 2) is used for the amplification of ovine BSE, natural (classical) scrapie also amplifies well in VRQ brain homogenate, and therefore should also amplify in VRQ cell lysate. Both the Rov9 cell line and tg338 mice express the VRQ ovine *PRNP* sequence, and tg338 brain homogenate is routinely used as PMCA substrate in our lab.

Figure 3-15 shows the comparison of two PMCA protocols for the amplification of natural scrapie with either Rov9 or tg338 brain homogenate. Protocol 1 was adapted from previously published PMCA experiments using RK13 cell lysate [395] and has a comparatively short sonication and incubation time, with more cycles completed over 24 hours. Natural scrapie (NS) amplified much more efficiently in tg338 brain homogenate than in Rov9 lysate. Both protocols produced limited amplification of NS in Rov9 substrate, demonstrated by the increase in signal in reactions seeded with 10^{-1} and 10^{-2} dilution of brain homogenate, compared to the unamplified reaction, which represents PrP^C converted by the NS seed.

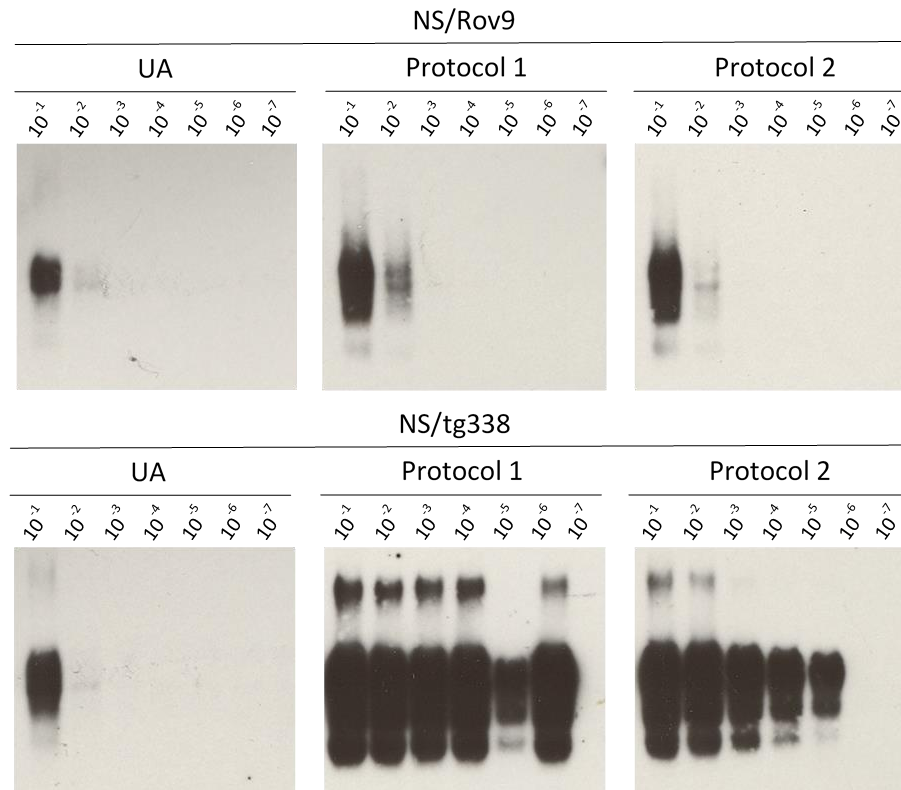


Figure 3-15: Amplification of natural scrapie with Rov9 lysate (top) and tg338 (ovine VRQ) brain homogenate (bottom) as substrate after one round of PMCA. Unamplified (UA) PMCA reactions are shown on the left. PMCA reaction products were PK digested and analysed by western blot using ROS-BC6. Protocol 1: 5 seconds sonication, 10 minutes incubation, 144 cycles, 50-60% amplitude. Protocol 2: 10 seconds sonication, 14 minutes 50 seconds incubation, 96 cycles, amplitude 60%. (5 minute exposure).

Figure 3-16 shows the amplification NS in Rov9 lysate, and provides a comparison between protocol one, and a third previously published protocol [282]. This protocol features a longer sonication and incubation, and an extended total reaction time at 48h. As in Figure 3-15, there is amplification of the NS seed using Rov9 lysate as substrate, and the two protocols are comparable in the level of amplification achieved. The increased amplification using cell lysate in Figure 3-16 compared to Figure 3-15 is likely due to differences in PrP^C levels between lysate batches. Previous PMCA attempts using low concentration Rov9 lysates $\sim >10\mu\text{g}/\mu\text{l}$ total protein failed to produce detectable amplification. Tg338 mouse brains overexpress PrP at 8-10x

physiological levels [151] which likely explains the much more efficient amplification of the seed using this substrate.

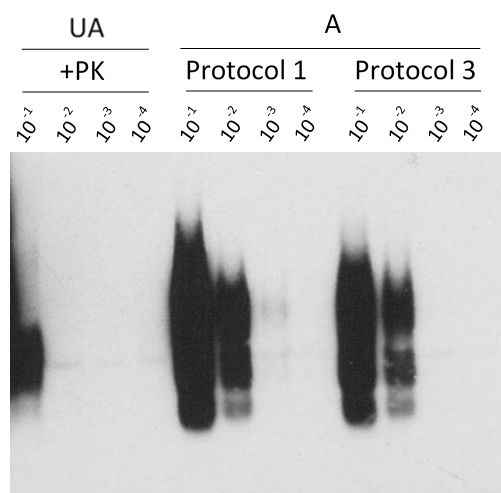


Figure 3-16: Amplification of natural scrapie with Rov9 lysate as substrate after one round of PMCA. Unamplified (UA) PMCA reactions are shown on the left. PMCA reaction products were PK digested and analysed by western blot using ROS-BC6. Protocol 1: 5 seconds sonication, 10 minutes incubation, 144 cycles, 50-60% amplitude. Protocol 3: 20 seconds sonication, 29 minutes 40 seconds incubation, 48h (cycles?), amplitude 65%. (1 minute exposure)

3.3.9.2 Preparation of CWD brain material for use as PMCA seed

Brain tissues from an experimental inoculation of white-tailed deer with CWD [399] were prepared as 20% homogenates, and diluted in either PMCA buffer for use in PMCA, or in PBS for NaPTA precipitation and western blotting. The prepared homogenates from white-tailed deer inoculated with elk (CWD elk) or mule (CWD mule) CWD inocula produced characteristic 3 band profile of proteinase-K resistant isoforms of PrP (PrP^{Sc}), which were comparable in molecular weight between the two samples. The negative control white-tailed deer had no detectable PrP^{Sc}. The positive control shows the slightly lower molecular weight of the unglycosylated band that is characteristic of BSE (Figure 3-17).

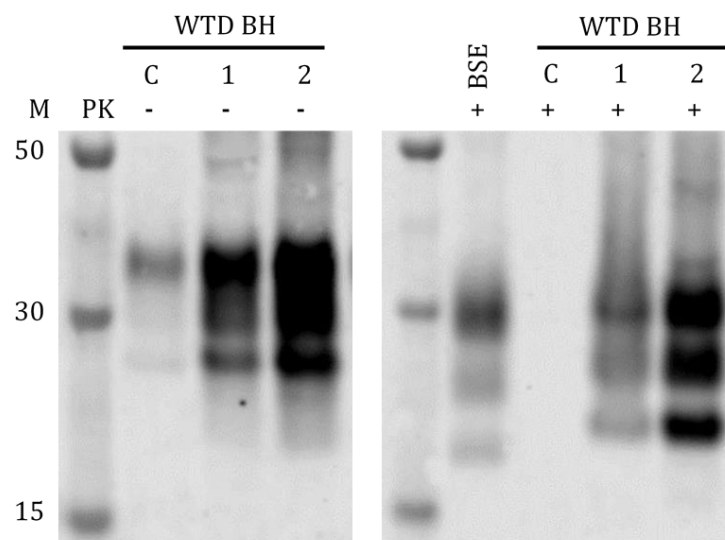


Figure 3-17: Western blot analysis of PrP^{Sc} in brains of experimental WTD, for use as seeds in PMCA.

10µl of NAPTA precipitated brain homogenate was loaded per lane. Molecular weights (kDa) are indicated on the left. Membrane was probed with anti-PrP antibody ROS-BC6. A BSE positive sheep medulla homogenate is used as a positive control for PK digestion. WTD brain homogenates (C) Negative control (1) WTD inoculated with Elk CWD (2) WTD inoculated with Mule deer CWD.

3.3.9.3 Testing of CWD seeds in PMCA

Initial testing of the CWD seeds was performed using the PMCA protocol 2 and both ovine VRQ (tg338) and ARQ (tgshpXI) PRNP substrates with no amplification detected. Unfortunately, transgenic mice expressing cervid PrP^C were not available, however we were able to source normal red deer brain homogenates. Theoretically, brain homogenate containing cervid PrP^C should be best suited for CWD amplification and would more closely resemble the PrP^C in our cell lysates. However, testing of the Elk CWD using protocol 1 failed to demonstrate amplification in either TPQ or TPE deer brain homogenate. This experiment was repeated with the addition of EDTA and heparin, which may be added to brain homogenates as co-factors to improve amplification in PMCA

[405], however both CWD seeds failed to amplify in deer brain homogenate (TPQ). Due to the prolonged storage of the CWD at -80°C, and the lack of amplification of these seeds in PMCA assays, CWD brain homogenate was tested in an alternative amplification assay. Real-time quaking induced conversion (RT-QuIC) assay using bank vole recombinant substrate showed efficient amplification of the CWD seed with short lag times at 10^{-2} to 10^{-4} dilutions (Performed by Lynne McGuire (Roslin Institute), results not shown), and confirmed that the lack of amplification in PMCA was likely due to the composition of the substrate, or lack of optimisation.

3.3.9.4 Optimisation of PMCA using cervid BSE

To further investigate whether poor amplification of CWD was due to issues with the seed or substrate, experiments were carried out using brain homogenate from red deer experimentally infected with BSE as an alternative seed. Amplification of cervid BSE in cervid homogenate substrate (TPQ) was limited to dilutions up to 10^{-2} , using protocols 1 and 3. The modified 48-hour protocol 2 produced only a slight improvement, of amplification to 10^{-3} (Figure 3-18). It may be expected that the cervid BSE seed would amplify most readily in substrate from the same species as the seed, however, sheep ARQ substrate has been previously demonstrated as a good substrate for amplifying BSE from cattle, sheep and pigs [406]. Cervid BSE was tested using tgShpXI brain homogenate (from transgenic mice expressing ovine ARQ) as substrate. Whilst in one initial experiment cervid BSE amplified only to a limit of 10^{-2} using protocol 1, a modified version of protocol 2 consisting of 192 cycles in 48h provided efficient amplification up to at least 10^{-8} after one round. These experiments would need to be repeated to determine if the increase in sensitivity was due to the change in protocol, or experimental error. Inefficient amplification in cervid brain homogenate may be due to lack of optimisation of the PMCA protocol for cervid substrate, however, is more likely due to the substrate itself. The level of PrP^C in cervid brain homogenates are lower than in Tg mouse brains (TgshpXI over-express ARQ at 2-4x)[151]. Additionally, the

normal deer brain homogenates were prepared from non-perfused brain samples. Blood is well documented as having inhibitory effects on PMCA amplification, and whilst measures were taken during the preparation of the homogenate to reduce blood contamination (3.2.9.1), any remaining blood may have inhibited PMCA amplification.

3.3.9.5 Cervid PRNP expressing cell lysate as substrate in PMCA

Limited preliminary PMCA experiments to amplify cervid *PRNP* expressing cell lysate seeded with CWD were unsuccessful. Western blotting of one experiment showed low levels of PrP expression detectable with both the 3F4 and ROS-BC6 antibodies, however no detectable amplification of the CWD seed. Both protocol 1 (published for the amplification of RK13 lysates in PMCA [395]) and protocol 2 were tested. The lack of amplification was likely due to the low levels of expression, as demonstrated by immunofluorescence and western blotting compared to Rov9. Improvement of expression levels for a highly concentrated lysate would be necessary to test and optimise a PMCA based assay using CWD seeds.

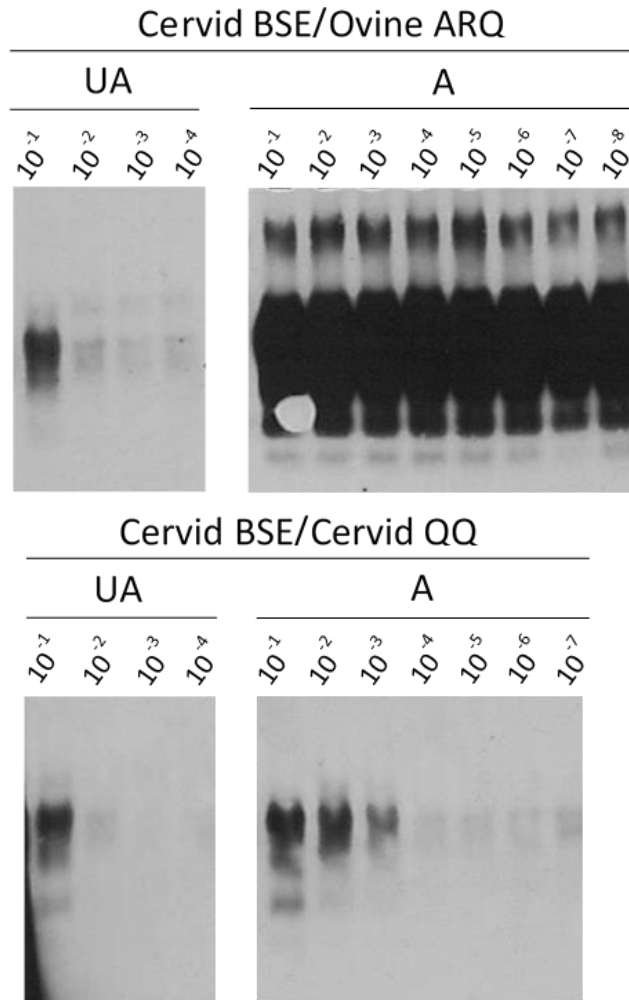


Figure 3-18: Amplification of cervid BSE using TgshpXI mouse (expressing ovine ARQ PrP^C) (top) or cervid (bottom) brain homogenate as substrate.

Unamplified PMCA reactions (UA) are shown on the left and amplified (A) on the right. One round of amplification as per PMCA protocol 2 (modified): 10 seconds sonication, 14 minutes 50 seconds incubation, 192 cycles (48h), amplitude 60%. (1 minute exposure).

3.4 Discussion

This chapter details work undertaken towards developing an *in-vitro* assay to assess the effect of cervid *PRNP* polymorphisms on CWD susceptibility. Whilst the initial work showed the potential for producing recombinant proteins of various cervid *PRNP* genotypes, focus was shifted towards developing a cell-lysate based PMCA method.

To develop a cell line expressing cervid PrP which could be used in cell-lysate PMCA assays, we initially began by attempting to replicate the methods used in a recent publication by Kurt *et al.*, 2017 [395]. In that paper, the pcDNA3.1 vector (Invitrogen) with mouse, human, bank vole or rabbit *PRNP* tagged with the 3F4 epitope was used to generate variants with site-directed mutagenesis, and used to transiently transfect RK13 cells, which allowed screening of 40 PrP variants for efficiency of conversion by PMCA and identification of residues that markedly impacted upon prion conversion. We aimed to generate various cervid *PRNP* variants using this method and measure amplification by PMCA when seeded with CWD. Percentage conversion (using densitometry) could be determined compared to the wild type sequence (TPQ) to allow a comparison of different British cervid *PRNP* polymorphisms on conversion efficiency in PMCA.

Expression of cervid prion protein in transiently transfected RK13 cells was successful, as was the incorporation of the 3F4 epitope, allowing specific detection of PrP^C derived from the cell line by the corresponding monoclonal antibody. Unfortunately, our attempts to recreate the previously published protocol did not produce high levels of PrP^C expression detected by western blot or immunofluorescence, despite varying a number of the experimental parameters in an attempt to optimise the procedure. The reason for the lower efficiency is unclear and may be due to unknown differences between our RK13 cell stocks, or reagents used in the procedure. Lipofectamine toxicity was often an issue despite using lipofectamine 3000, which is specifically formulated for low toxicity.

I also attempted a stable transfection of RK13 cells, reasoning that this approach

may improve expression levels though the positive selection of RK13 cells expressing cervid PrP^C and produce a cell line that could be maintained over multiple passages. Again, the expression levels achieved were very low, with few highly expressing cells identified on immunofluorescence. Due to the low numbers of positive cells, it was unlikely further cloning would produce a cell line with higher expression levels.

Successful amplification of the Rov9 lysate (expressing ovine VRQ) in PMCA was achieved using natural scrapie as a PrP^{Sc} and three different protocols, with minimal differences observed between protocols. Amplification in normal VRQ brain homogenate substrate (tg338) was much more efficient than in cell lysate, with detection after 1 round of PMCA at 10⁻⁵ or 10⁻⁶. Cell-based PMCA assays previously published have shown that the efficiency of amplification is dependent on the total protein concentration in the cell lysate [79, 397], and we also noted slightly increased sensitivity when using more concentrated Rov9 lysate. Generating high expression levels in cervid PrP cell lines, at least to levels comparable with Rov9, was therefore of vital importance for producing lysate for PMCA substrate.

Rov9 is a stably transfected RK13 cell line with the tetracycline Tet-On expressing system, a pTRE plasmid into which the PrP open reading frame is subcloned, and the pTet-On plasmid, which encodes the rtTA regulator protein required for tetracycline inducible expression. A puromycin resistance plasmid is co-transfected into cells to allow for selection. We replicated the procedure used to generate the Rov9 cell line [385] by construction of a pTRE vector containing the cervid TPQ open reading frame with the 3F4 epitope. Initial transfection attempts were successful and showed expression of cervid PrP^C in RK13 cells. Transfection and puromycin selection for stably transfected cells was attempted using a range of different protocols. In the most effective protocol ('GB') clusters of highly expressing cells were identified on a background of weakly expressing cells. Based on this experiment, stable transfection with the pTRE vector was more successful than with the pcDNA3.1 vector. Further cloning and subcloning of this cell line improved levels of

expression further, however even the sub-clone (SC5) with the highest levels of PrP^C expression did not reach the levels seen in Rov9 cells on western blot, and only had clusters of highly expressing cells visible by immunofluorescence. The reasons for this difficulty in generating highly expressing cell lines is unclear. It may be that expression of the vector produces a competitive disadvantage, making it more likely that lower or non-expressing cells will outcompete. Alternatively, it may be that more extensive subcloning (with larger numbers of clones) would be necessary to select out a single clone expressing at the high levels identified in clusters of cells in sub-clone 5. Previous publications describing the creation of RK13 cell lines with the pTRE plasmid have variable numbers of clones tested, from just 9, to 55 [384, 385, 392]. Other reports for generating PrP^C expression in RK13 cells rely on flow-assisted cytometry sorting (FACS) to generate single cell transfectant clones [396]. Future work on this cell line could therefore involve either further subcloning or FACS sorting as a way of sorting the highly expressing cells from our sub-clones showing variable expression levels, and generate a homogenous population of highly expressing cells comparable to Rov9. Of note, previous RK13 cell lines expressing cervid *PRNP* have required stable co-transfection with a plasmid expressing HIV gag precursor protein to enable persistent prion infection [393, 407]. Alternatively, a recent paper has shown the use gene-edited murine cell lines (CAD5 and MEF) for the propagation of CWD, using CRISPR-cas9 to ablate endogenous PrP expression and lentiviral transduction to reconstitute cells with mouse, bank vole or cervid PrP genes [408]. This approach appeared to avoid the extensive single-cell subcloning necessary for expression and permissibility of cell lines to infection with prions. Production of a stable cell line permissive to CWD infection would allow examination of the effect *PRNP* polymorphisms on susceptibility in cell infection assays [409], as well as providing a means for production of PMCA substrate.

As the cell lysate PMCA could not progress further without adequate deer PrP expressing substrate, we hoped to optimise and develop positive controls for PMCA by first testing CWD seeds in other substrates. Preliminary attempts to

amplify CWD in sheep PrP^C homogenates were unsuccessful, which is not surprising given the species barrier; CWD does not transmit readily in ovinised transgenic mice [268] and sheep only show evidence of transmission after intracerebral inoculation in low numbers, and after prolonged incubations [267]. As our goal was to use deer-expressing PrP^C cell lysates, we prepared normal brain homogenates from red deer (TPQ and TPE genotypes) to use as a PMCA substrate for optimisation. Surprisingly, CWD seeds failed to amplify in this substrate. We next tested cervid-BSE, as the PMCA protocol in our lab was developed for sheep BSE. Cervid BSE amplified in both sheep ARQ and deer TPQ substrates, however, was much more efficient in the former. This in combination with the failure to amplify CWD seeds suggested the problem was more likely to be due to the substrate, either due to lower expression levels in deer brains compared to the transgenic overexpressing sheep ARQ lines, or possibly due to contamination by blood which is known to be inhibitory in PMCA [84, 189, 201, 410]. Whilst transgenic mouse brains are more commonly used to provide PMCA substrate, sheep brain homogenates prepared similarly to our normal deer brains have been previously used for PMCA substrate [411]. However, other reports of PMCA using cervid brain homogenates as PMCA substrates were from animals perfused with PBS at the time of death, suggesting this may be necessary to prevent inhibitory blood contamination [230]. Equally, this lack of amplification may be due to the lack of optimisation for CWD in our PMCA system. Differing protocols and the addition of heparin and EDTA to the substrate did not improve amplification. CWD has previously posed challenges for amplification in PMCA, with inconsistent amplification, and mouse-adapted mCWD replicates poorly compared to mouse adapted scrapie stains RML, ME7 and 227 (Patricia Aguilar Calvo, pers. comm.). Future attempts to optimise CWD-PMCA in our lab could employ larger reaction volumes and additional Teflon beads – where our protocols used a single bead, previous published protocols report efficient amplification of CWD in PMCA using two [87] or three [155] Teflon® beads and larger reaction volumes.

Future PMCA experiments could also include a dilution series using Rov9 substrate, to determine the optimal concentration of lysate to be used as substrate. Comparison of Rov9 and the cell line expressing cervid PrP by PNGase treatment, western blotting and densitometry would show comparative expression levels in each cell line and allow estimation of the total protein concentration necessary for use as a PMCA substrate. Further optimisation of the PMCA protocol may include testing of various co-factors. Brain homogenate from PrP null mice has previously been used to supplement the substrate in cell lysate PMCA [79], however this supplementation was not necessary when the lysate concentration was high. This study also described the use of large multilayer cell culture flasks to create the bulk culture necessary to produce highly concentrated substrates for PMCA [79].

An alternative approach may be possible using RT-QuIC assays, which has been successfully developed in our lab for detection of PrP^{Sc} in sheep blood since the recombinant protein work described at the start of this chapter was undertaken. The constructs for production of North American and British cervid recombinant PrP described could now be used to produce large batches of recombinant protein by a new method and used as substrate in RT-QuIC assays. This approach has already been applied to North American cervid *PRNP* polymorphisms, where using the rate of amplification and amyloid extension efficiencies as metrics to compare different recombinant substrates produced results that aligned for the most part with *in vivo* susceptibility [301].

In summary, this chapter describes the work undertaken to develop *in-vitro* assays for the assessment of the effects of cervid polymorphisms on CWD susceptibility. Whilst much of this work remains preliminary, we have shown 1) the expression and purification of recombinant cervid prion protein for use in fibrillation assays (and potentially RT-QuIC), 2) the use of site-directed mutagenesis to create vectors for the expression of UK red deer and fallow sequences in the pTRChis vector (3) the use of site-directed mutagenesis to introduce the 3F4 epitope into cervid *PRNP* sequences (4) the expression of cervid PrP^C in RK13 cells using two different vector systems, and the specific

detection of this protein using the 3F4 antibody (5) subcloning of stably transfected RK13 cells to identify clones with high levels of PrP^C expression and (6) the use and characterisation of a cell lysate (Rov9) as a substrate for the amplification of natural scrapie in PMCA.

4 Primary transmission of CWD isolates from Norway and Canada in wild-type mice.

4.1 Introduction

The experimental passage of prion disease isolates into new host species has been used to investigate fundamental questions in prion disease, for example prion transmission, prion replication and dissemination, species barriers and zoonotic potential, and prion strain diversity [31, 143, 412]. Prion strains emerge upon primary transmission and serial passaging of an isolate in a species different from the original host, most commonly in small rodents. Prion strains are operationally defined by the differences in heritable phenotype under controlled transmission [412]. How an agent devoid of any genetic material can encipher specific strain properties is an important question in prion research, and current evidence suggest that strain characteristics are encoded in the specific misfolded conformation of the protein (which may be impacted by host genetics), as well as the presence/absence of specific co-factors needed for conversion [41, 144, 152–155]. Strain adaptation is believed to occur during replication in new hosts, or *in-vitro* environments where a different PrP^C environment drives an adaptive response with selection of a dominant strain or the generation of novel strains [155]. In newly recognised prion diseases such as CWD in Norway, strain characterisation is of importance as there may be strain-specific differences in pathogenesis, environmental persistence, resistance to decontamination, and importantly, potential for cross-species transmission.

Research undertaken in the 1960's and 70's demonstrated the propagation of distinct disease phenotypes in panels of inbred mice following experimental challenge with isolates of scrapie [413, 414]. These early experiments formed the basis for strain typing experiments, the vast majority of which have been carried out in rodent models such as mice, hamster and bank voles [135, 322, 412]. In Edinburgh, strain typing experiments have conventionally been carried out in wild-type mice panels, which employ inbred mouse lines VM, RIII and C57BL/6. The different genetic backgrounds of these mice with RIII and C57BL/6 expressing *Prnp*-a (Leu-108, Thr-189), and VM *Prnp*-b (Phe-108, Val-

189), produce varied phenotypes (such as different incubation periods) upon experimental passage of disease [146]. In cases where there is poor or no transmission to wild-type mice the development of transgenic mice expressing the *PRNP* gene of various host species has been a useful addition.

A number of different transgenic models expressing cervid PrP have been developed, and are reviewed in [151]. In North America, two strains of CWD have been identified in white-tailed deer upon passage to ferrets [321], hamsters [322], and transgenic mice expressing cervid PrP [149, 318]. In elk, one [268, 322, 328] or two [149] strains appear to be propagated. In both white-tailed deer and elk of specific genotypes (132L and 116A respectively) there is experimental evidence of further strains provided by transgenic mouse studies [302, 323]. CWD strains are discussed in depth in section 1.6.6. Since the emergence of CWD in Norway in 2016 it has therefore been a priority to assess the strain properties of CWD identified in affected cervid populations. Strain typing of Norwegian cases may provide suggestions of the origins of European CWD, as well as answering important questions about inter-species transmissions and zoonotic potential. A considerable number of transmission experiments of the European CWD isolates in experimental models including mice, transgenic mice, hamsters and bank voles are ongoing (summarised in [344]). Recent reports of transmissions of Norwegian isolates in bank voles provide evidence that the strains in Norwegian cervid populations are different from those in North America, and different between reindeer and individual moose cases [346].

Whilst there are various *in-vitro* methods which also provide evidence of prion strains (section 1.5), bioassay remains as the gold standard [146]. Experimental passage of TSE inocula in rodents is undertaken typically by either intracerebral or intraperitoneal inoculation (or a combination of both). Transmissions to wild-type mice are typically characterised by incubation period of between four months to over two years, followed by a clinical phase with progressive clinical signs [415]. Traditionally, the emphasis has been placed on the incubation period and vacuolation profile for distinguishing between strains.

On primary passage incubation periods are generally extended and very variable. Repeated passage under controlled experimental conditions stabilises strain properties and produces incubation periods that are highly reproducible and affected by both mouse genotype and strain. The order of incubation period in VM, RIII and C57BL/6 mice, known as 'incubation ranking' is a measure used to aid strain differentiation. Clinical signs may also contribute to the strain phenotype, whether due to the specific nature of the signs, such as the 'hyper' (HY) and 'drowsy' (DY) phenotypes of TME in hamsters [416], or the presence or absence of signs in wild-type mice, such as upon transmission of human vCJD and sCJD respectively [9].

Vacuolation scoring is a semi-quantitative assessment of the severity of vacuolation in 6 grey and 3 white matter brain regions (see Figure 4-2 and Table 4-3). When an average of these results is plotted for each mouse line, a 'lesion profile' is produced, which is highly characteristic and reproducible for different strains. The lesion profile was particularly important for the recognition of BSE in other species, due to the unusually stable strain characteristics which produced consistent lesion profiles regardless of the species from which the inocula originated (cattle, humans or other species)[417].

The biochemical analysis of infected brain tissue through PK digestion and western blotting, in combination with immunohistochemistry, is required for definitive diagnosis of prion disease. Detection of PK-resistant protein is denoted PrP^{Sc} throughout this chapter. Typically, PrP^{Sc} has a triple banding pattern composed of nonglycosylated, monoglycosylated and diglycosylated PrP, with molecular masses of approximately 20, 25 and 30 kDa respectively after protease digestion. The conformational differences between prion strains are reflected in biochemical differences that may aid in the differentiation of strains; these differences include the PK cleavage site (producing differently sized bands on western blot), glycosylation ratio, relative PK resistance, unfolding characteristics in guanidinium hydrochloride, appearance on electron microscopy, differences in the secondary structure assessed using FTIR Fourier

Transform Infrared spectroscopy, and activity in the conformation-dependent immunoassay (CDI) [155, 159, 412, 418, 419]. However, it is important to note that biochemical differences between strains are not always readily apparent; CWD1 and CWD2 strains in North American cervids were indistinguishable by western blotting and had similar unfolding characteristics [149].

Neuropathological analyses include histological vacuolation scoring, abnormal prion protein deposition detected by immunohistochemistry (denoted PrP^D - throughout), and other neuropathological markers. Due to the requirement of multiple passages in mice before stabilisation of a strain phenotype based on incubation period and lesion profile, attention has recently focused on the use of detailed immunohistochemical analysis of PrP^D profiles to differentiate strains after primary passage [420, 421]. PrP^D deposition patterns may be a powerful tool to discriminate prion strains, even with mixtures of prion strains, or in a few individuals after primary passage [148, 420–423]. Astrocytic and microglial activation are also identified in prion disease, detected by morphological changes and the upregulation of markers such as glial fibrillary acidic protein (GFAP) for astrocytes, and Iba1 for microglia. Alternatively, it has recently been shown that astrocytic marker CD44 and its splice variant (v6) are upregulated in strain-specific patterns, which may also be used to differentiate strains in experimental mice [424].

The lymphoid tropism of prion strains is an interesting aspect of prion transmission, although is often not the focus of analysis during primary transmission. Lymphoreticular accumulation of prion protein varies in natural prion diseases, with relatively little in BSE and atypical scrapie, but is much more prominent in horizontally transmitted prion diseases like scrapie and CWD. In cervids with CWD, lymphoid involvement may vary, with less abnormal PrP deposition in elk compared with deer [327]. Interestingly, lymphoid involvement is a prominent feature of Norwegian reindeer with CWD, but absent in the moose and red deer cases [17, 343, 345]. Similar differences are also seen in experimental transmissions such as hamsters infected with HY or DY strains of TME. The HY strain is characterised by widespread PrP^D

throughout nervous, lymphoreticular and skeletal muscle tissues, as well as bodily fluids, whereas this is limited to only the CNS of DY infected hamsters [412]. In addition to strain or genotype related differences in lymphoid replication of prions, lymphoid tissue may be more permissible to cross-species transmission or harbour different prion strains to the brain [108, 425–427].

In this chapter, I describe the pathological phenotypes in the brains of wild-type mice following primary passage of CWD isolates from both Norway and Canada. I also examine if lymphoid replication is an important feature of primary passage of CWD in wild-type mice. The use of wild-type mice allows for comparison with a large catalogue of strain typing studies previously performed in Edinburgh (Neuropathogenesis Unit, Institute of Animal Health/Roslin Institute). This chapter includes the primary passage of Canadian CWD isolates to provide a comparison for the Norwegian isolates, and the opportunity to compare results between labs based at the University of Edinburgh and the University of Alberta, Canada.

4.2 Materials and methods

All animal studies were approved by the local ethical review board and conducted according to the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986 under PPL 70/8552 and P0BC76F9C.

4.2.1 *Norwegian and Canadian CWD isolates*

Norwegian reindeer and moose CWD isolates were provided for passage in wild-type mice as part of a larger strain-typing study. Information about the isolates is summarised in Table 4-1. Norwegian cervid brain material was provided by Dr Sylvie Benestad (Norwegian Veterinary Institute, Oslo, Norway). The reindeer isolate was from a free-ranging reindeer, the first natural case reported in this species, and the first case of CWD in Europe. A sample of brain cortex was received, previously reported to be both IHC and ELISA (TeSeE, Bio-Rad) positive [17]. The moose tissue provided (ID P138 or 'Moose No1') was the first detected moose case in Norway. The tissue provided consisted of a section of midbrain; testing of the obex of this animal was positive with both IHC and ELISA (TeSeE, Bio-Rad) [343]. Further details of both cases are summarised in section 1.6.7.2. Canadian CWD isolates were provided by Dr Debbie McKenzie (University of Alberta, Canada). The first isolate provided was from an experimental transmission of CWD into a white-tailed deer with the H95/S96 genotype [234]. The second isolate was from a hunter-harvested mule deer, collected in the Swift Current area of Saskatchewan in 2008.

Table 4-1: Geographical location, species, origin, age and PRNP genotype of the CWD affected cervids studied by transmission to wild-type mice.

Location	Species	Origin	Age	PRNP genotype	Thesis ID	Alternative ID	Ref
Norway	Moose	Wild	13y	KK ₁₀₉ MM ₂ 09	Nor-M	16.60.P138 M-NO1 A918	[343]
Norway	Reindeer	Wild	3-4y	VV ₂ GG ₁₂₉ S S ₁₃₈ VV ₁₆₉ NN ₁₇₆ SS ₁₂₅	Nor-R	16-04-V142 R-NO1 A817	[17]
Canada	White-tailed deer	Captive (experimental)	4+y	H95/S96	Can-WT	1297	[234]
Canada	Mule deer	Wild	n/a	wt/wt	Can-M	74128	n/a

4.2.2 Inoculation, clinical scoring, and tissue collection

4.2.2.1 CWD inocula preparation

Whole brain material from Norwegian cervids (reindeer cortex and moose midbrain) were processed to a 10% brain homogenate in sterile saline solution in a dedicated derogated Containment Level (CL) 3 laboratory at the Roslin Institute. Brain material from positive CWD cases in Canada was provided as a 20% homogenate and was further diluted in sterile PBS to provide a 10% homogenate. Bacterial contamination of the inocula was assessed by routine microbiology, as Canadian isolate Can-M was positive, the homogenate was pasteurised at 80°C and sonicated for 1 minute prior to inoculation.

4.2.2.2 Inoculation of experimental mice

Experimental transmission of CWD inocula was carried out in mice maintained in derogated CL3 facilities in the Biological Research Facility (BRF), Roslin Institute. Three inbred wild-type mouse lines were used: RIII, C57BL/6J (*Prnp*-a genotype) and VM (*Prnp*-b genotype). C57BL/6J were sourced from Charles River, and VM/RIII were available inhouse (Roslin Institute). Groups (n=18) were age (6-8 weeks) and sex matched where possible.

Mice were treated with 2.8mg amoxicillin clavulanate (Synulox® RTU) prior to anaesthesia and inoculation was performed by appropriately trained staff. Norwegian isolates were inoculated intracerebrally (20µl) into the right midline of the skull, and intraperitoneally (100µl). Due to lack of available material, Canadian isolates were inoculated intracerebrally (20µl) only.

4.2.2.3 Clinical monitoring and scoring

Clinical scoring was carried out in accordance with previously established TSE scoring systems [111]. Trained BRF staff were blinded to mouse line and CWD inoculum. Mice were monitored daily, and formally scored once per week. Clinical signs were broad due to the novel nature of the CWD isolates, and early clinical signs included non-specific changes such as changes to behaviour (e.g. hyperactivity) or a poor condition coat, progressing to more specific clinical signs including ataxia, kyphosis, weight loss. The clinical scoring system is outlined in Table 4-2. Mice were sacrificed humanely by a schedule 1 method upon the following conditions: (i) two consecutive scores of definitely affected (+ or G) (ii) after receiving scores of definitely affected in 2 of 3 weeks, (iii) significant deterioration of condition or welfare concerns, or (iv) study end-point.

Table 4-2: Clinical scoring system for TSE infected mice

Clinical score	Description
1	Normal mouse – No evidence of disease
2	Early clinical disease symptoms
g	Early symptoms accompanied by alteration of gait
+	Terminal/end point of disease
G	Terminal/end point of disease accompanied by severe deterioration of gait

4.2.2.4 Incubation period or survival time

Incubation or survival time was calculated as the period in days between initial inoculation (date of injection) and the date at which mice were sacrificed at

clinical end point (incubation period) or end of study (survival time). Mean incubation periods were calculated with standard error of the mean (SEM) for each individual group based on inocula and mouse strain.

4.2.2.5 Post-mortem tissue collection

Whole brains were removed and divided longitudinally by a parasagittal cut to the left of the midline. The right half was fixed in 10% formal saline for histology. The left half was flash frozen in liquid nitrogen before storage at -80°C for biochemical analysis. Spleens were also halved and treated the same way.

4.2.2.6 Analysis exclusion criteria

Mice were removed from analyses upon the following conditions: (i) they were culled at ≤ 100 dpi (before the start of clinical scoring), (ii) pathological examination could not be carried out due to poor condition of the brain (autolysis or damage).

4.2.3 Histological analysis of mouse tissues

4.2.3.1 Fixation, embedding and sectioning of mouse brain for histological techniques

Processing of tissues onto slides was provided by experienced personnel in the Easter Bush Pathology service. Briefly, collected tissues were fixed in 10% formal saline for a minimum of 48 hours, decontaminated by immersion in 98% formic acid for 1.5 hours [428] (to lower effective prion titre for health and safety), coronal sections of mouse brain taken (see Figure 4-1) and embedded in paraffin wax. Sections were cut at a thickness of 6 μ m and mounted onto Superfrost™ (H&E) or Super frost-plus™ (IHC) glass slides (ThermoFisher), dried at 37°C overnight and stored at room temperature.

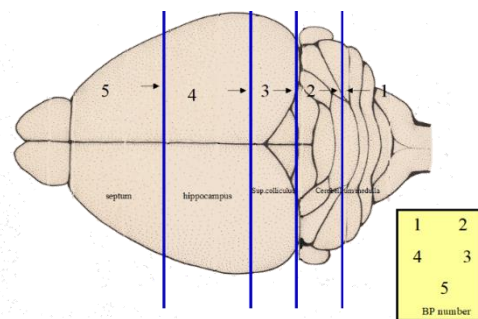


Figure 4-1: Location of the four rostro-caudal levels taken to produce coronal sections in the fixed mouse brain. Arrows indicate the face-up direction of the section. (Image from SOP – Roslin Institute)

4.2.3.2 Vacuolation scoring and lesion profiles

The level of vacuolar pathology in 9 grey and 3 white matter brain areas was assessed according to a standard scoring system by experienced personnel blinded to study details [414]. The semi-quantitative system scored grey matter from 0 to 5, and white matter from 0 to 3 (see Table 4-3 and Figure 4-2)

Table 4-3: Criteria for vacuolation scoring in mice.

Grey matter	Description
0	No TSE vacuolation or signs of TSE in that area
1	An occasional hole possibly indicative of aging or TSE vacuolation, inconclusive diagnosis
2	Definite signs of TSE vacuolation: oval-shaped holes, clustering, no even spread of TSE vacuolation
3	Even spread of TSE vacuolation throughout the area
4	Heavier, even spread of TSE vacuolation throughout the area and the vacuoles are beginning to coalesce to form large vacuoles
5	Very heavy even spread of TSE vacuolation throughout the area and frequent large vacuoles. Lace-like appearance
White matter	
0	No TSE vacuolation or signs of TSE in that area
1	Definite signs of vacuolation in white matter
2	Even spread of vacuolation throughout the white matter
3	Very heavy even spread of vacuolation throughout the white matter with vacuolation coalescing to form large vacuoles

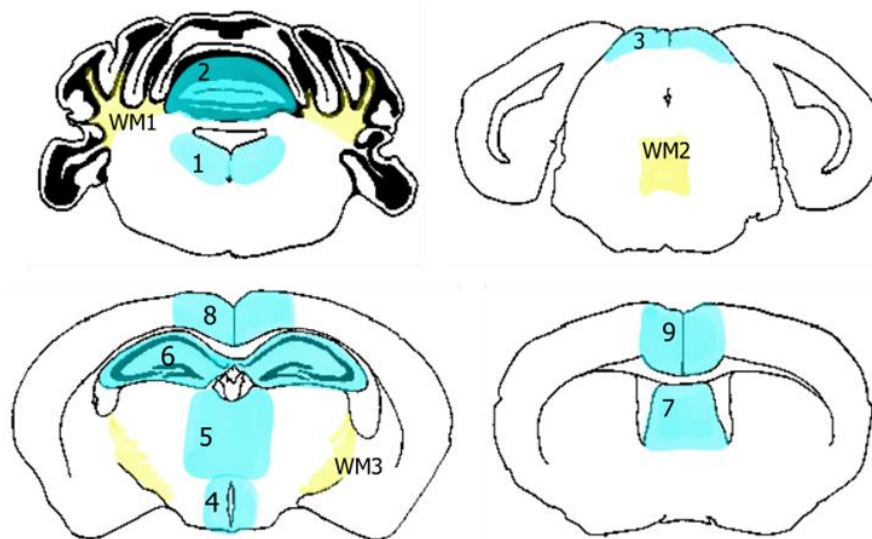


Figure 4-2: Schematic diagram of the coronal sections and scoring regions used in lesion profiling.

Grey matter areas (blue): 1 – medulla, 2-cerebellum, 3- superior colliculus, 4- hypothalamus, 5-thalamus, 6- hippocampus, 7- septum, 8- retrospinal cortex, 9-cingulate and motor cortex. White matter areas (yellow): WM1– cerebellar peduncle, WM2– decussation of superior CP/tegmentum, WM3- basal cerebral peduncle/pyramidal tract. (Image adapted from Boyle et al 2017)

4.2.4 Immunohistochemical analysis of brain tissues

Sections were de-paraffinised in xylene followed by rehydration in graduations of absolute alcohol to water using an XL Autostainer (Leica Biosystems, Newcastle upon Tyne, United Kingdom). Sections were pre-treated by autoclaving at 121°C for 15 minutes in 0.1M citric acid buffer and were cooled slowly to room temperature in water. Sections were immersed in 98% formic acid for 5 minutes, followed by brief stepwise incubations in 70%, 95% and 99% alcohol. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 15 minutes. Sections were washed in water, then incubated in PBS/BSA wash buffer for 5 minutes. Sections were blocked with a 1:20 dilution of normal rabbit serum (NRS) for 20 minutes. Anti-PrP specific primary antibody 6H4 (Prionics™)[429] was diluted 1:1000 in wash buffer to give a final concentration of 1.9µg/ml, and incubated on sections overnight. For each animal, brain sections were stained in duplicate, with 6H4 and a control with either NRS or an irrelevant IgG1 isotype control antibody.

Sections were incubated in wash buffer 3 times at 5 minute intervals, then incubated with secondary biotinylated rabbit anti-mouse antibody for 1 hour. Sections were incubated in wash buffer 3 times at 5 minute intervals. Biotinylated antibodies were detected using horse radish peroxidase-conjugated to the avidin-biotin complex (Vectastain^R ABC elite kit, Vector Laboratories, Peterborough, United Kingdom) as per manufacturer's instructions. Sections were incubated in wash buffer 3 times at 5 minute intervals. Staining was visualised using 3,3'-diaminobenzidine (DAB) (Merck KGaA, Darmstadt, Germany) reagent with 200µl hydrogen peroxide added immediately before use, incubated for 5-10 minutes to give an appropriate intensity. Sections were counterstained in haematoxylin Z, and Scott's tap water, before dehydration. Sections were mounted using an automated glass coverslipper (Eprelia CTM6, Thermo Scientific). Slides were scanned using a Nanozoomer XR (Hamamatsu) and imaged using NDP.view2 software. Mice were analysed in a blinded study for PrP^D deposition, with record of staining types and locations noted. Additionally, each brain was subjectively scored from + to +++, where + = minimal staining present in a few brain areas, ++ = minimal staining that is widespread, or intense staining in restricted areas, and +++ widespread intense staining.

4.2.5 Immunohistochemical analysis of spleen tissues

Sections were treated as described above with the following changes: autoclaving was performed in distilled water, sections were blocked with normal goat serum (NGS), immunostained with anti-PrP specific mAb (Clone ROS-BH1, final concentration 0.36 µg/ml, Roslin TSERC, [430]) and biotinylated goat anti-mouse secondary antibody, detected using Vectastain ABC (see above) and visualised using Vector[®] NovaRED[®] peroxidase substrate kit (HRP) (SK-4800, Vector Laboratories). Detection was achieved using NovaRED rather than DAB substrate to provide easy discrimination from haemosiderin often present in spleen samples.

4.2.6 Thioflavin staining of amyloid

Paraffin-embedded sections were dewaxed and rehydrated and incubated in 1% thioflavin-S solution (Sigma) for 5 minutes. Sections were differentiated in 70% industrial denatured alcohol (IDA) for 3 x 1 minute incubations and washed twice in distilled water, and once in PBS/BSA wash buffer. For counterstaining, slides were incubated Rabbit anti-bovine GFAP (DAKO, GA52461-2) for 1 hour at room temperature, washed 3 x 5 minutes in wash buffer, then incubated with goat-anti-rabbit Alexa Fluor™ 594 (1:1000) for 1 hour at room temperature. Slides were washed 3 x 5 minutes in wash buffer and once in distilled water, and mounted using fluorescent mounting medium (DAKO). Images were captured using a confocal laser scanning LSM880 Airyscan microscope using the 488 and 594 tracks and processed using Zen software.

4.2.7 Biochemical analysis of mouse tissues

4.2.7.1 Homogenisation of mouse tissues

All work involving experimental mouse tissues was carried out in a derogated CL3 TSE laboratory. Tissues were removed from -80°C storage and onto ice. In aseptic conditions, tissues were weighed and transferred to 2ml Lysing matrix D tubes containing ceramic beads (MP bio, 116913050). Cold 1X dPBS buffer without calcium and magnesium (Gibco, 14190094) was added; 1000µl for half brains, and 500µl for spleens. Tissues were homogenised using a Precellys 24 homogeniser, with two 20 second bursts of 5000rpm, and a 5 second incubation. The homogenate was removed, and the beads washed with the remaining volume of PBS required to dilute sample to a 10% homogenate. Samples were centrifuged at 2000 x g for 5 minutes to pellet cellular debris, and the supernatant removed for analysis.

4.2.7.2 PK treatment and western blotting

10% brain homogenates were treated with 50µg/ml proteinase K (PK) (Sigma #P6556) at 37°C for 1 hour. Following this, 200µl brain samples were

centrifuged at 16100 x g for 1 hour at 4°C, to concentrate and pellet PrP^{Sc}. The pellet was then re-suspended in NuPage™ LDS Sample Buffer (4x) (Invitrogen™, NP0008) supplemented with reducing agent (Invitrogen™, NP0009), and denatured at 95°C for 5 minutes. Electrophoresis and semi-dry western blotting was carried out as described in section 3.2.6. Briefly, proteins were separated by electrophoresis, and transferred to PVDF membrane. The membrane was blocked using LICOR blocking buffer, and incubated with primary antibody (6H4, 0.38µg/ml), and secondary antibody goat anti mouse IRdye 680RD (Licor Biosciences, 926-68070), before detection using LICOR odyssey.

4.2.7.3 NaPTA precipitation of spleen homogenates

Sodium phosphotungstic acid (NaPTA) precipitation is a way to capture and concentrate PrP^{Sc} to increase the sensitivity of immunoblotting [431]. Due to the variation in published protocols, the NaPTA procedure was compared between a 'long' protocol, which included treatment with an endonuclease (benzonase) to reduce sample viscosity and incubation with NaPTA/MgCl₂ solution overnight. The 'short' protocol omitted the benzonase digestion and reduced incubation time to 30 minutes. In both protocols, PK digestion was carried out prior to NaPTA precipitation to avoid altered migration of PrP^{Sc} fragments that may be associated with NaPTA [432]. The short protocol was effective in preliminary experiments and is detailed below.

Samples were thawed at room temperature and sonicated to mix. All incubations were performed at 37°C with constant shaking. For a 100µl sample (10mg tissue equivalent), 100µl (1:1) of 4% sarcosyl/PBS was added and incubated for 10 minutes. PK (1mg/ml) (Sigma #P6556) was added to a final working concentration of 50µg/ml and incubated for 1 hour. PK digestion was stopped by addition of 2.2µl of phenylmethylsulfonyl fluoride (PMSF) and vortexing every minute for 5 minutes. Pre-warmed NaPTA/MgCl₂ was added to a final concentration of 0.3% w/v and incubated for 30 minutes. Samples were centrifuged at 15800 x g for 30 minutes at room temperature, then the supernatant removed from pellet of precipitated PrP^{Sc}. Pellets were washed

with 40µl 0.1% w/v sarcosyl/PBS and 40µl 250mM EDTA, vortexed to mix and centrifuged as above for 30 minutes. Supernatant was removed, and if the pellet had white precipitate (residual MgCl₂), a further wash with 80µl 250mM EDTA was performed. Pellets were air dried before resuspension in 12µl of 0.1% sarcosyl/PBS. NaPTA precipitated samples were stored at -20°C prior to western blotting or RT-QuIC experiments.

4.2.8 RT-QuIC analysis

Full-length bank vole recombinant (residues 20-230, M109, provided by Lynne McGuire), was thawed on ice and passed through two 100KDa filters (Amicon) by centrifugation at 4°C to remove any precipitates. Protein concentration was determined by Nanodrop™ (Thermo Fisher). All components of the substrate were filtered using Millex-GP 0.22µm syringe filters, (Merck). RT-QuIC reaction mix was prepared as follows: 10mM phosphate buffer (pH 7.4), 170 mM NaCl (total 300mM including phosphate buffer), 10µM Thioflavin-T (ThT), 10µM ethylenediaminetetraacetic acid tetrasodium salt (EDTA), and 0.1 mg/mL recombinant PrP^c (Bank Vole 23–230). Brain and spleen homogenates (10%) were prepared as described previously (4.2.7.1). Homogenates were vortexed and diluted (10-2, 10-3 and 10-4) in PBS buffer. NaPTA precipitated spleen samples (4.2.7.3) were resuspended in 0.1% sarcosyl/PBS and diluted further in PBS to produce the tissue equivalent of 10-1, 10-2 and 10-3 dilutions.

Samples were vortexed and 2µl of the appropriate brain or spleen dilution, or 2µl of PBS for unseeded negative control, was used to seed 98µl RT-QuIC reaction mix to give a total 100µl final reaction volume. Reactions were performed in quadruplicate for each sample in a 96-well optical flat bottom plate (Thermo Scientific). Plates were sealed with sealing film (Thermo Scientific) and incubated in a FLUOstar® OMEGA plate reader (BMG LABTECH Ltd.) at 42°C for >90h with intermittent shaking cycles: double orbital with 1 minute shake (700rpm), 1 minute rest. ThT fluorescence measurements, expressed in arbitrary units (relative fluorescent units (RFU)), were taken every 15 minutes using 450nm (excitation) and 480nm (emission) wavelengths, with

a bottom read. The fluorescence reading (RFU) for each quadruplicate sample was recorded against time, a baseline reading of ~39000 RFU was recorded for unseeded and negative samples, with positive samples reaching saturation at 260,000 RFU. To achieve representative sampling along the baseline, five fluorescence readings from unseeded and 8 negative control samples at 5, 20 and 35 hours were selected. A threshold value of 55,000 RFU was chosen based on the baseline mean +10SD (39976+13552). A cut-off at 45 hours was chosen due to the increase in spontaneous fibril formation seen in unseeded and negative control samples from 45 hours onwards. Samples were considered positive if two or more replicate wells surpassed the threshold before the cut-off at 45 hours. Borderline samples were defined as samples with two wells surpassing the threshold by 45 hours, with lag times of greater than 25 hours.

4.3 Results

4.3.1 *Clinical features, incubation period and lesion profiling of CWD transmissions in wild-type mice*

Transmission of Canadian and Norwegian CWD isolates to wild-type mice produced evidence of clinical or subclinical prion disease based on pathological (TSE vacuolation and/or abnormal PrP^D deposition) and a highly sensitive prion detection assay (RT-QuIC). Of the four isolates, Can-WT produced the highest numbers of mice with convincing clinical and neuropathological evidence of prion disease, with 40% of mice positive for clinical disease increasing to 72% of recipient mice having positive neuropathology. Numbers of IHC positive mice were highest in the C57BL/6 line (94%), and slightly lower in VM (64%) and RIII (53%). In all three lines, vacuolation and clinical signs were detected in mice that also showed PrP deposition on IHC. In contrast, the other three isolates (Nor-R, Nor-M and Can-M) did not readily transmit in any of the mouse lines, and showed more inconsistent evidence of prion infection (see Table 4-4 and Table 4-5).

4.3.2 Survival time and clinical signs

During primary passage of a new prion isolate, survival time/incubation period is often extended compared to subsequent passages, a feature of overcoming the species barrier. Only once adapted and stabilised, is comparison of survival time/incubation period a useful feature of strain typing. The low attack rates seen upon transmission of Nor-R, Nor-M and Can-M isolates also makes useful comparison difficult. Mice displaying positive signs of transmission after inoculation with these isolates were surviving approximately 600 days.

A single mouse inoculated with the Nor-M isolate displayed clinical signs including changes to gait for approximately 1 month prior to cull, with the final clinical score indicating a severe deterioration of gait and a loss of body condition. Clinical signs were a common feature of transmission of the Can-WT isolate (see Table 4-2). Mice with overt clinical signs displayed kyphosis, ataxia, torticollis and head bobbing, as well as more general signs of poor condition including rough coat and weight loss and glazed or dull eyes. Due to the novel nature of the two Norwegian isolates, clinical parameters had to be fairly wide to account for unusual clinical signs, however this increases the chance of misinterpretation of age-related clinical behaviour changes. This is likely the reason for mice scored clinically positive upon transmission of the Can-M isolate (VM and RIII mice) but with no observable pathological signs of disease.

Table 4-4: Primary transmission of Canadian wild mule deer (Can-M) and experimental white-tailed deer (Can-WT) to wild-type mice

Inocula	Mouse details			Transmission results ¹				
	Strain	Total	Excluded	Survival time ²	Clinical suspects ³	Vacuolation (dpi)	IHC (dpi)	WB (dpi)
Can-M	VM	18	2	247-670 (551±34)	6/16 (611±20)	1/16 (670)	0/16	0/15
Can-M	C57BL/6	18	0	506-674 (640±14)	0/18	0/18	0/18	0/13
Can-M	RIII	16	1	239-679 (562±39)	1/15 (528)	0/15	0/15	0/15
Can-WT	VM	11	0	105-596 (487±54)	7/11 (558±12)	6/11 (559±14)	7/11 (568±12)	4/4
Can-WT	C57BL/6	17	0	519-674 (638±11)	7/17 (624±20)	13/17 (634±13)	16/17 (636±11)	4/4
Can-WT	RIII	15	0	304-658 (488±34)	3/15 (519±21)	2/15 (540)	8/15 (551±49)	4/4

¹Expressed as number positive/total number, (dpi) or if over 3 (average dpi±SEM) ²Range in dpi, (average±SEM). ³Mice scored clinically positive with + or G on final clinical scoring.

Table 4-5: Primary transmission of Norwegian reindeer (Nor-R) and moose (Nor-M) isolates to wild-type mice

Inocula	Mouse details			Transmission results ¹				
	Strain	Total	Excluded	Survival time ²	Clinical suspects ³	Vacuolation (dpi)	IHC (dpi)	WB (dpi)
Nor-R	VM	18	0	574-600 (593±2)	0/18	1/18 (574)	0/18	0/18
Nor-R	C57BL/6	17	0	412-601 (583±11)	0/16	1/17 (574)	2/16 (596, 601)	0/17
Nor-R	RIII	16	1	119-606 (494±43)	0/15	0/15	0/15	0/15
Nor-M	VM	16	0	517-600 (590±6)	0/16	0/16	0/16	0/16
Nor-M	C57BL/6	18	0	469-601 (590±7)	0/18	0/18	0/18	1/18 (601)
Nor-M	RIII	15	1	138-600 (465±43)	1/14 (575)	1/14 (575)	0/14	1/10 (575)

¹Expressed as number positive/total number, (dpi) ²Range in dpi, (average±SEM). ³Mice scored clinically positive with + or G on final clinical scoring.

4.3.3 Vacuolation/Lesion profiles

TSE associated vacuolation was assessed in a blinded study by an experienced technician (Aileen Boyle, Roslin Institute). Consistent with previous experimental transmission studies of North American CWD to wild-type mice, vacuolation was not a major feature (Diack, pers. comm.). Three mice inoculated with the Norwegian isolates were marginally vacuolation positive, with evidence of low-level vacuolation (scoring level 1, see Table 4-3), mainly in the granular layer of the cerebellum and/or thalamus. This included two mice inoculated with the Nor-R isolate and a further mouse from the Nor-M transmission, which also displayed clinical signs (see Table 4-5). Similarly, transmission of the Can-M isolate produced only one marginally vacuolation positive mouse, with vacuolation in the medulla and cerebellum. Vacuolation was observed more frequently in mice inoculated with the Can-WT isolate, in line with higher transmission rates. Vacuolation was generally low to moderate with vacuolation scores of ≤ 2 . VM (6/11) and C57BL/6 (16/17) lines had sufficient numbers of affected mice to produce lesion profiles (see Figure 4-3), whereas RIII (2/15) did not. VM mice showed higher vacuolation scores in the medulla and thalamus compared to other scoring areas, whereas C57BL/6 mice

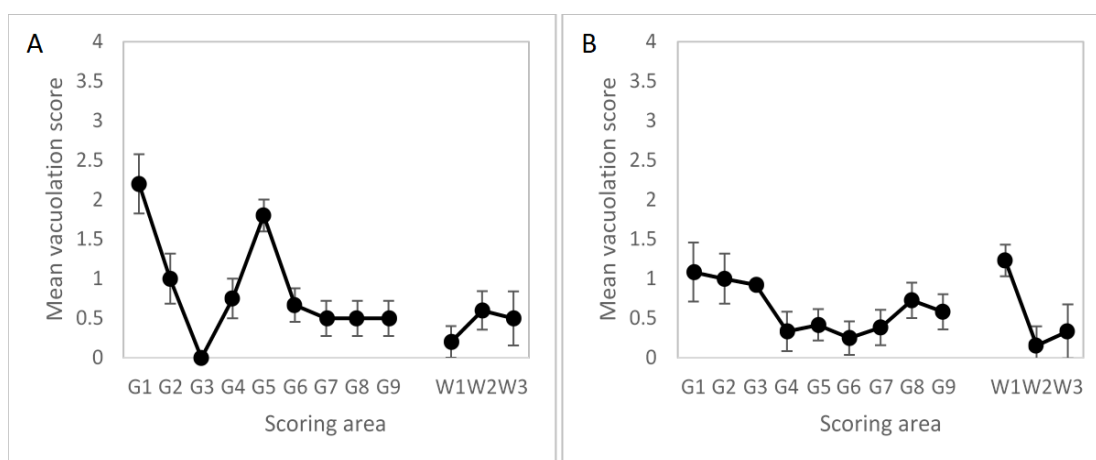


Figure 4-3: Lesion profiles in VM (A) and C57BL/6 (B) mice after primary passage with Can-WT isolate. Data points represent the mean $\pm$ SEM of ≥ 5 mice per group. G1-medulla, G2-cerebellum, G3- superior colliculus, G4-hypothalamus, G5-thalamus, G6-hippocampus, G7-septum, G8-retrospinal cortex, G9-cingulate and motor cortex. WM1-cerebellar peduncle, WM2-decussation of superior CP/tegmentum, WM3-basal cerebral peduncle/pyramidal tract.

showed generally low grade vacuolation across all areas. Outwith the scoring areas, vacuolation was also noted in the olfactory tract, habenular nucleus and cochlear nucleus.

Typical histopathological and immunohistochemical findings are shown in Figure 4-4. Vacuolation and significant gliosis is shown in (c), and areas that were often targeted, such as the thalamus, showed a correlation between vacuolation and PrP^D deposition (described in next section).

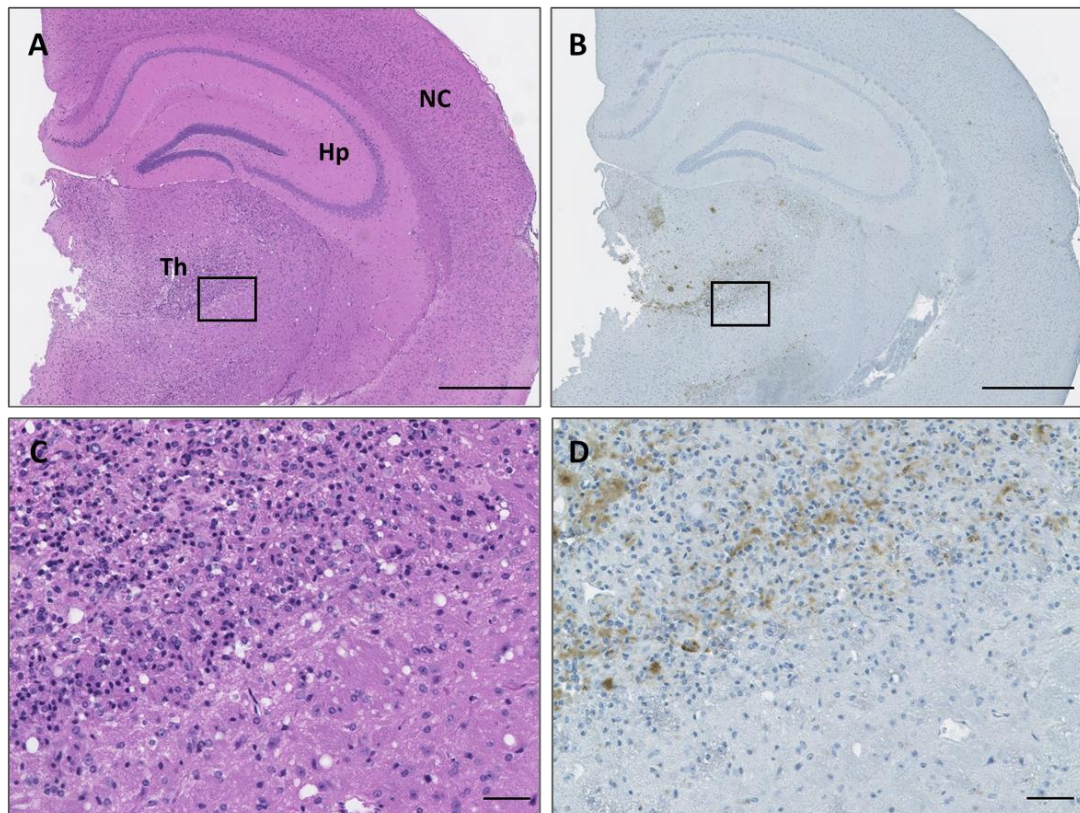


Figure 4-4: Histopathological and immunohistochemical analysis of a VM mouse inoculated with the Can-WT isolate.

Sections showing thalamus (Th), hippocampus (Hp) and neocortex (NC), stained with haematoxylin and eosin (A) and by immunohistochemistry for PrP^D with 6H4 antibody (B) Magnification of the ventral thalamic nucleus (box) is shown in (C) and (D). Definite signs of TSE vacuolation including oval shaped holes and clustering (vacuolation score 2) are shown in (C), as well as significantly increased cellularity due to gliosis in this area. Correlation with PrP^D is shown in (D). Scale bars: A,B 500µm, C,D 50µm

4.3.4 Immunohistochemical detection of prion protein deposition in the brains of wild-type mice

Immunohistochemical (IHC) detection of abnormal prion protein deposition (PrP^D) is a technique widely used in the diagnosis of both experimental and naturally occurring TSEs. The detection of accumulated misfolded prion protein by specific anti-prion antibodies is a marker of prion disease and occurs early in the disease process. In natural TSEs, different PrP^D deposition patterns and spatial distributions in the brain are dependent on both agent strain and host genetics (PrP genotype) [433, 434]. In reported experimental transmissions of prion disease in small rodents, PrP^D is often shown by PET blotting, which has the advantage of showing protease-resistant misfolded protein PrP^D, but removes much of the finer neuro-anatomical detail that may be detected by IHC. Recent studies have shown that detailed analysis of prion protein deposition patterns in the brains of wild-type mice may be used for differentiation of common laboratory strains, or discrimination ovine or bovine BSE sources [420, 421]. The benefits of such analysis include that it may be effective after only primary passage, and in low numbers of mice. Therefore, this section details PrP^D staining patterns and neuroanatomical distribution for the primary transmission of CWD isolates in wild-type mice.

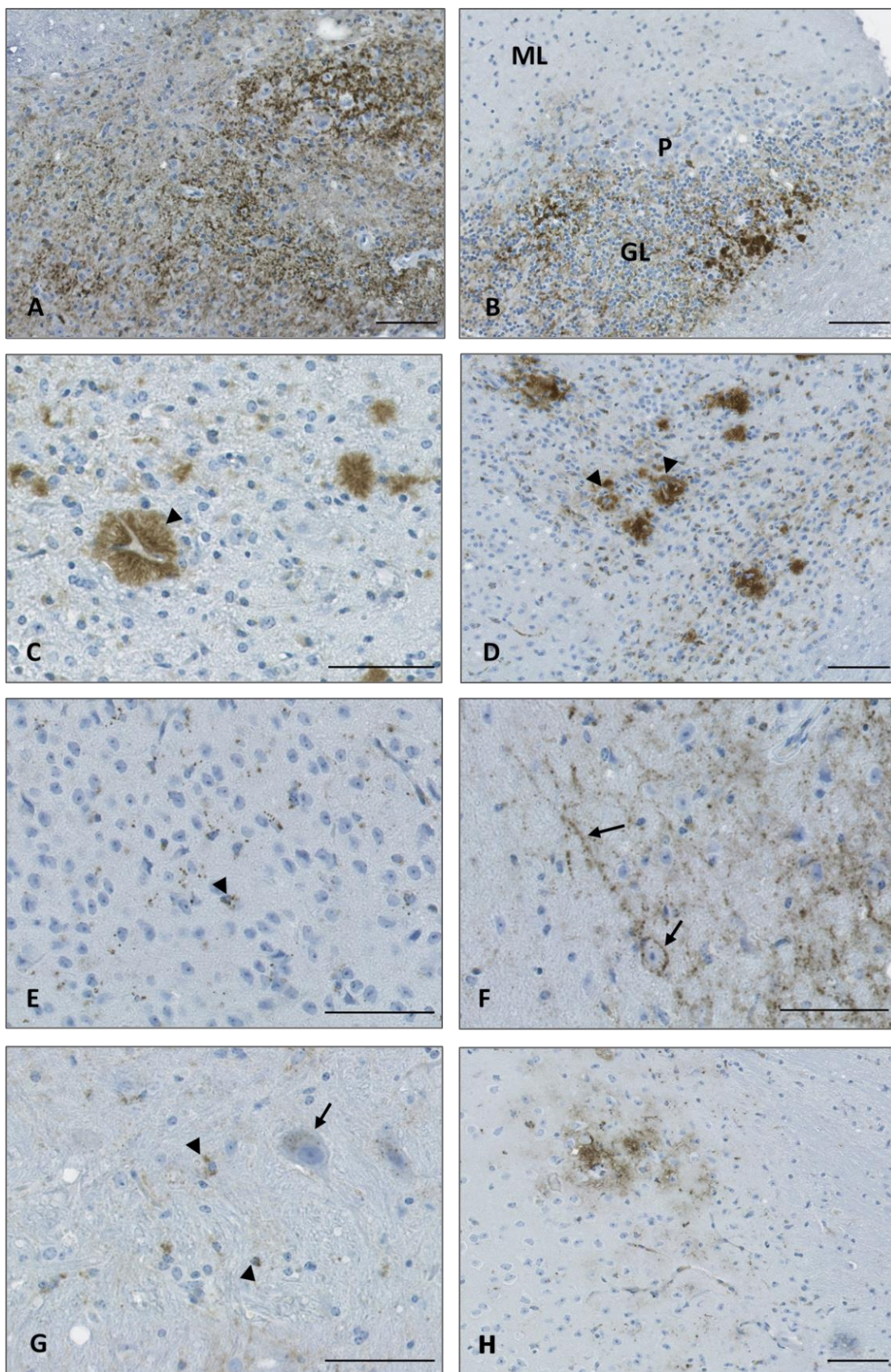
4.3.5 Classification of PrP^D distribution patterns and staining types

Mice were analysed for PrP^D deposition in a blinded study. The range of different PrP^D deposition patterns observed in wild-type mice after inoculation with CWD are shown in Figure 4-5. Due to the higher numbers of mice positive by IHC, this section describes deposition patterns after inoculation with the Can-WT isolate, and this terminology will be used throughout to describe PrP^D types identified in CWD-infected mice. Coarse granular staining was a common pattern identified and appeared as focal accumulations of particulate PrP^D deposits throughout the neuropil on a background of diffuse staining (Figure 4-5A). Often this staining was strong and accompanied by aggregates, which

were focal dense accumulations of PrP^D which varied in size (Figure 4-5B). Large aggregates were also referred to as disorganised plaques. A variety of other plaque types were identified, including perivascular (Figure 4-5C,D), subependymal (Figure 4-6B) and sub-pial. Often plaques appeared in clusters, with smaller aggregates and larger disorganised plaques surrounding fibrillar plaques, which have characteristic bundles radiating amyloid fibrils. Plaques were thioflavin positive, highly organised and unicentric with bundles of radiating amyloid fibrils (see Figure 4-6), or more disorganised and multicentric. Punctate staining was a common finding and featured fine discrete depositions of PrP^D throughout the neuropil (Figure 4-5E), sometimes associated with linear or 'tracking' patterns which followed fibre tracts (Figure 4-5F). In areas with fine punctate or coarse granular staining, intraglial deposition was also a common feature, with dense deposits larger than the punctate staining visualised in close association with glial cell nuclei (Figure 4-5E,G). In rare cases, staining appeared as small diffuse radiating deposits which were reminiscent of stellate deposition often seen in ruminant species (Figure 4-5H). Perineuronal deposition was identified by granular deposits surrounding the neuronal perikarya (Figure 4-5F), and was more commonly noted than intraneuronal deposition, which was very rarely identified (Figure 4-5G).

Figure 4-5: PrP^D distribution patterns in wild-type mice inoculated with CWD.

A,B,E,F,G,H: Can-WT isolate in C57BL/6, C,D: Can-WT isolate in VM mice. (A) Coarse granular staining in the cochlear nucleus, medulla. (B) Coarse granular staining in the granular layer (GL) of the cerebellum, with aggregations. Fine punctate staining surrounding the purkinje cells (P) and in the molecular layer (ML). Varying sizes of perivascular plaques (arrowheads) in the thalamus (C) and neocortex (D). Plaques may be organised and fibrillar (C) or disorganised/diffuse and associated with coarse granular staining (D). Intraglial staining with prominent granules was often visualised (arrowheads, E,G). (E) Fine punctate staining in the hypothalamus. (F) Perineuronal and linear tracking (arrows) staining in the substantia nigra. Less common staining patterns included (G) intraneuronal (arrow) staining in the neuronal cell body of the vestibular nuclei of the medulla and (H) Stellate staining in the striatum. Scale bars 50µm.



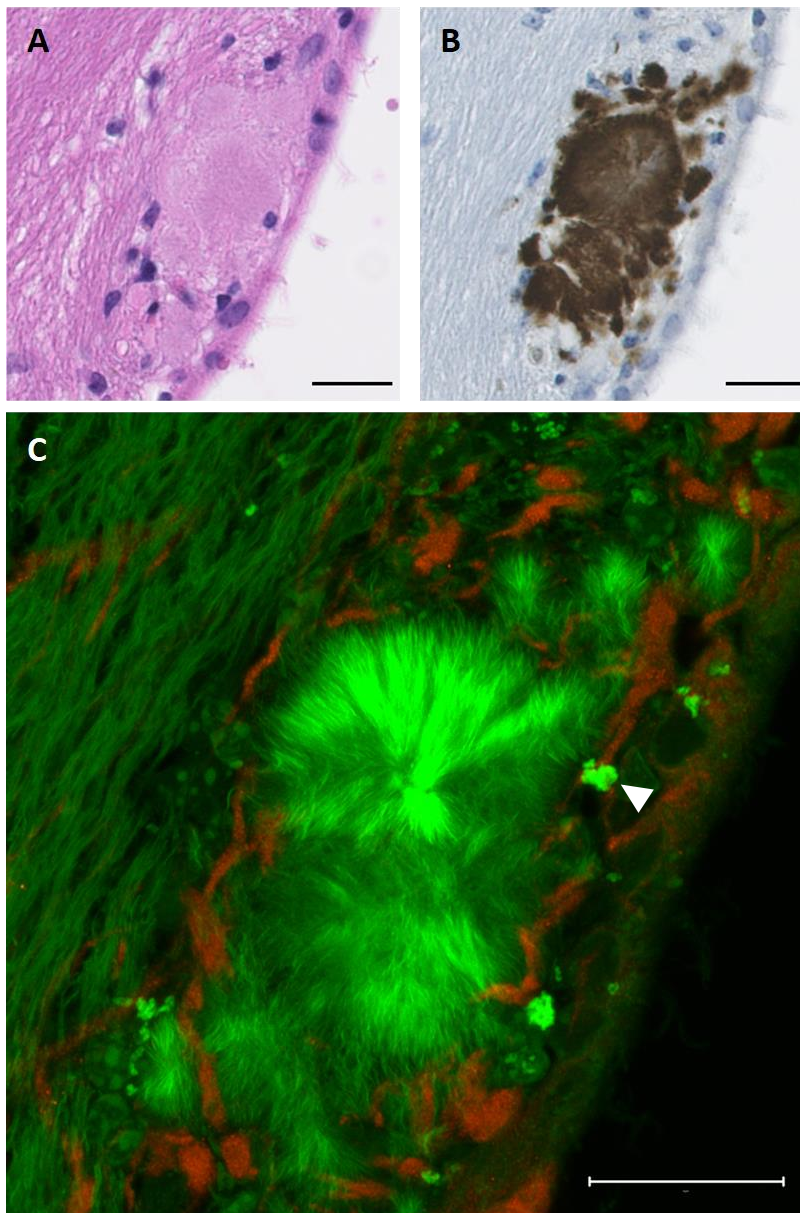


Figure 4-6: Plaque characterisation in a C57BL/6 mouse inoculated with Can-WT isolate (M00132817).

This accumulation of fibrillar plaques and granular deposition was located in the corpus callosum adjacent to the lateral ventricle.

(A) H&E: plaques appear as pale fibrillar eosinophilic areas of neuropil (B) Anti-PrP antibody 6H4 (C) Fluorescent staining using thioflavin-S (green) highlights amyloid plaques of varied size characterised by bundles of radiating amyloid fibrils as well as granular deposition (arrowhead). Rabbit-anti-bovine Glial fibrillary acidic protein (GFAP) (1:300) was used to provide counterstaining of astrocytes (red), with astrocytic processes surrounding PrP deposition. Fluorescence images were captured using confocal laser scanning Zeiss LSM 880 airyscan microscope and processed using zen software. Scale bar 20µm.

4.3.6 Transmission of Canadian CWD isolates

The transmission of Can-WT isolate to wild-type mice resulted in a number of mice with evidence of PrP^D deposition and a variety of deposition patterns and locations which varied between mouse strains. In contrast, PrP^D was not identified in any mice inoculated with the Can-M isolate. Overall, the most common staining patterns were fine punctate to coarse granular deposition, with aggregates and plaques. Plaques were a common feature and varied depending on mouse strain/line. The regions most commonly affected were the

Table 4-6: Overview of IHC positive mice inoculated with Can-WT isolate.

Mouse details			Transmission results		
Strain	ID	Survival (Days)	Clinical suspect	Vacuolation	PrP ^D
VM	M00130312	596	-	+	+
	M00130315	535	+	+	+
	M00130316	596	-	+	+
	M00130861	534	+	+	+
	M00130863	576	+	+	+
	M00130873	517	+	+	+
	M00130874	595	+	-	+
RIII	M00131450	658	-	-	+
	M00131452	658	-	-	+
	M00131453	658	-	-	+
	M00131454	540	+	+	+
	M00131459	304	-	-	+
	M00131464	393	-	-	+
	M00131465	540	+	+	+
	M00131467	658	-	-	+
C57BL/6	M00132814	658	-	+	+
	M00132815	519	+	+	+
	M00132816	616	+	+	+
	M00132817	616	+	+	+
	M00132818	616	+	+	+
	M00132824	667	+	+	+
	M00132825	609	-	+	+
	M00132826	667	+	+	+
	M00132827	667	+	+	+
	M00132832	591	-	+	+
	M00132833	674	-	+	+
	M00132834	674	-	+	+
	M00132835	674	-	+	+
	M00132836	674	-	-	+
	M00132842	674	-	-	+
	M00132843	582	-	-	+

thalamus, medulla and substantia nigra (midbrain). Specific nuclei targeted include the habenular, vestibular, cochlear, and cerebellar nuclei, and the locus coeruleus. Unlike other mouse-adapted prion strains, the hippocampus was not an important area of PrP deposition.

4.3.6.1 PrP deposition patterns vary between VM, C57BL/6 and RIII mice inoculated with Can-WT isolate

The numbers of mice with PrP^D deposition was highest in the C57BL/6 mouse line with 94% of mice testing positive, followed by VM mice with 64%, and RIII with 53%. In line with this, deposition was often more pronounced and widespread in C57BL/6 and VM mice compared to RIII.

Features of PrP^D deposition in VM mice included more frequent plaque formation, comparative to C57BL/6 and RIII mice. Plaques were often perivascular and varied in size, with regions affected including the striatum and cortex, thalamus, medulla and cerebellum, with the thalamus particularly affected. In C57BL/6 and RIII mice, plaques were less common and, when present, they tended to form focal depositions of fibrillar plaques surrounded by smaller disorganised plaques and aggregates. These plaques were often located just beneath the ependymal cell layer alongside a ventricle (sub-ependymal) or underneath the meninges (sub-pial). In all mouse lines, occasional large disorganised plaques were identified in the corpus callosum or external capsule. Differences in staining patterns were particularly evident in the thalamus (Figure 4-7) where VM mice had widespread plaque deposition with aggregates and coarse granular deposition, particularly in the ventral thalamic nuclei. C57BL/6 and RIII mice showed predominantly fine punctate to coarse granular and intragial staining particularly in the dorsal lateral geniculate nucleus (dLGN) and also the ventral thalamic nuclei. Granular deposition in the fasciculus retroflexus and optic tract was also a feature in C57BL/6 mice. All three lines often had staining ranging from fine punctate to granular in the habenular nucleus of the thalamus, and rarely had any staining in the

hippocampus, with only a few mice showing sparse fine punctate deposition in this area.

In all mouse lines, fine punctate staining was often present throughout affected brain regions, with coarse granular and aggregated deposition in more heavily affected regions such as the dorsal raphe nucleus and substantia nigra of the midbrain, and the vestibular and cochlear nuclei of the medulla. When visualised, the locus coeruleus was also a focal area for granular deposition. Whilst the cerebellar nuclei were often an area of deposition with fine punctate and intragial staining in all three mouse lines, the patterns of deposition varied in the cerebellar cortex (Figure 4-7). In VM mice, the cerebellar cortex was often free of staining, with infrequent focal areas of fine punctate to coarse granular staining in the granular layer and sometimes extending into the molecular layer. In animals with particularly heavy PrP^D deposition, aggregates and plaques were identified in all three layers of the cerebellar cortex. Compared to other mouse lines the granular layer of the cerebellar cortex was particularly affected in C57BL/6 mice, with coarse granular deposition commonly observed throughout this layer. Heavy deposition was sometimes associated with a loss of granule cells and thinning of this layer. As PrP^D deposition was generally more mild in RIII mice, staining in the cerebellar cortex was often not observed. However, in those mice with heavier deposition elsewhere in the brain staining was observed as fine punctate to granular deposition particularly in the white matter (arbor vitae), with minimal staining in the granular and molecular layers. Representative images of the prion protein deposition in all brain areas of three mouse lines inoculated with Can-WT isolate are shown in the appendix (Figure 7-1, Figure 7-2, Figure 7-3).

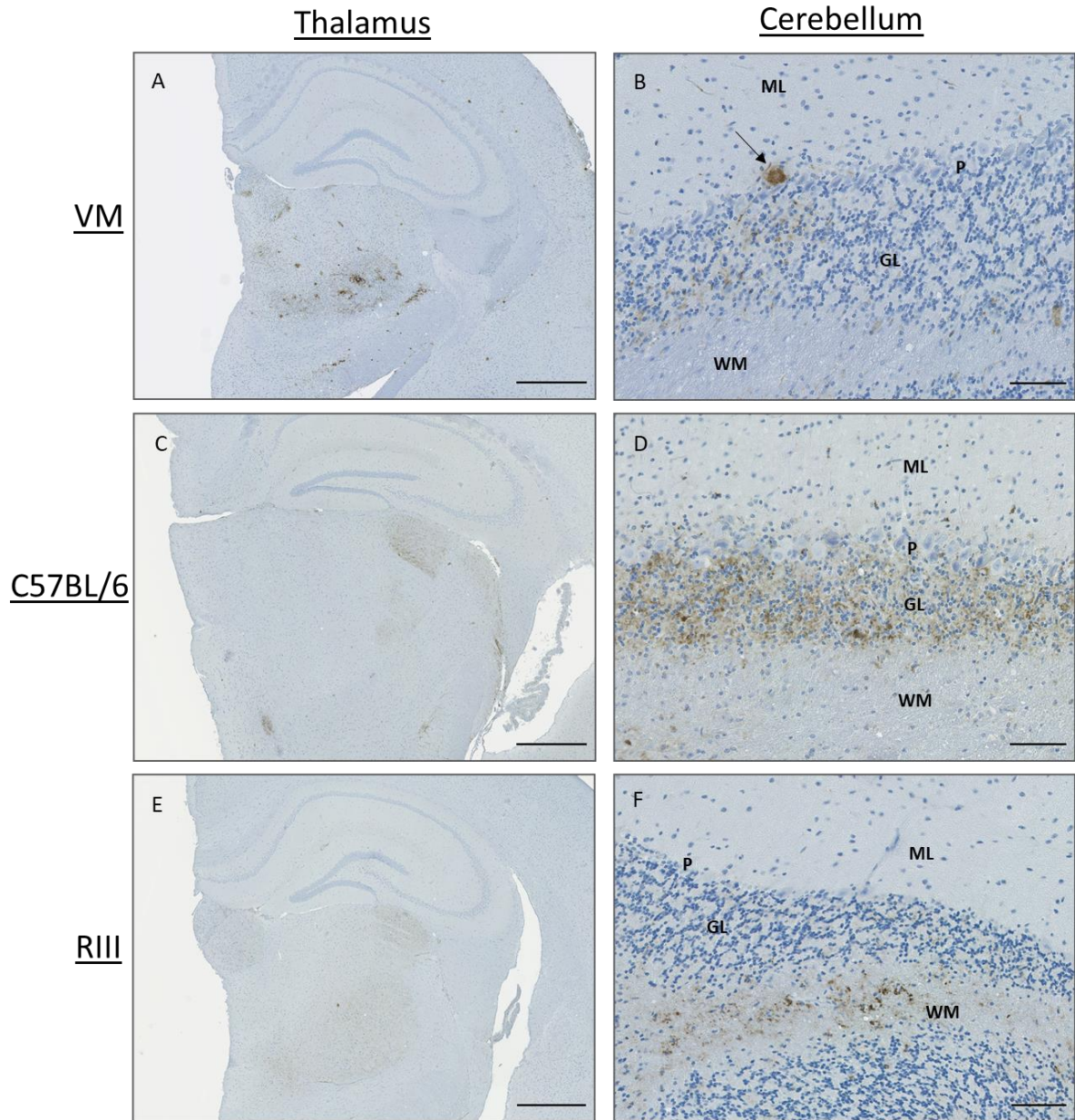


Figure 4-7: Comparison of PrP^D deposition in the thalamus and cerebellum of wild-type mice inoculated with Can-WT isolate.

Layers of the cerebellum as follows: molecular layer (ML), purkinje cell layer (P), granular layer (GL) and white matter (WM). (A) M130873: Fine punctate to coarse granular staining with frequent aggregates and perivascular plaques in the thalamus and to a lesser extent the neocortex. (B) M130861 Focal patches of fine punctate to granular deposition with occasional plaques (arrow), not restricted to any particular layer. (C,D) M132825: (C) Fine punctate and intragial staining in the ventral thalamus with coarse granular staining in the dLGN and fasciculus retroflexus. (D) Coarse granular deposition restricted to the GL of the cerebellum. (E,F) M131454 (E) Fine punctate, intragial and diffuse staining in the ventral thalamus, particularly in the dLGN. (F) Fine punctate, occasionally granular staining restricted to the WM, with minimal staining in cerebellar cortex (GL,P,ML).

4.3.7 Transmission of Norwegian CWD isolates

Out of a total 100 mice inoculated with Norwegian isolates, 97 brains were suitable for analysis. Only 2/49 mice inoculated with Nor-R had evidence of low levels of PrP^D deposition (Table 4-7), whilst 0/48 mice inoculated with Nor-M were positive. To ensure a good representation of staining, analysis was repeated on positive mice on at least three separate coronal sections.

Table 4-7: IHC positive mice from Norwegian transmission experiments

Mouse details				Transmission results		
Isolate	Strain	ID	Survival (Days)	Clinical suspect	Vacuolation	PrP ^D
Nor-R	C57Bl/6	M00111889	596	-	-	+
Nor-R	C57Bl/6	M00111925	601	-	-	+

4.3.7.1 PrP^D deposition in Nor-R inoculated mice

Two mice had evidence of PrP^D deposition at 600 days post-inoculation, without convincing evidence of vacuolar pathology or clinical signs. This was consistent with the very limited deposition of PrP^D observed. Both IHC positive mice were C57BL/6. In one mouse, PrP^D staining was present and associated with needle track damage in the right cerebral cortex at the level of the corpus callosum. There was a focal large disorganised plaque (Figure 4-8F) and fine punctate staining (Figure 4-8E) following direction of the needle track. No staining was identified in other regions of the brain. More extensive staining was identified in a second mouse, with a similar large disorganised plaque present in the right cortex, (Figure 4-9C). Fine punctate and intragial staining was observed in the habenular nucleus (Figure 4-9D) of the thalamus. Moderate levels of fine punctate to coarse granular staining was present in the medulla, which was aligned sub-ependymally beneath the fourth ventricle (Figure 4-9G,L) and occasional disorganised plaques were also observed (Figure 4-9H). A focal area of fine punctate to coarse granular staining was identified in the locus coeruleus (Figure 4-9I). The locations and staining types identified in these C57BL/6 mice are reminiscent of the deposition seen in C57BL/6 inoculated with Can-WT isolate, however larger numbers would be necessary for comparison.

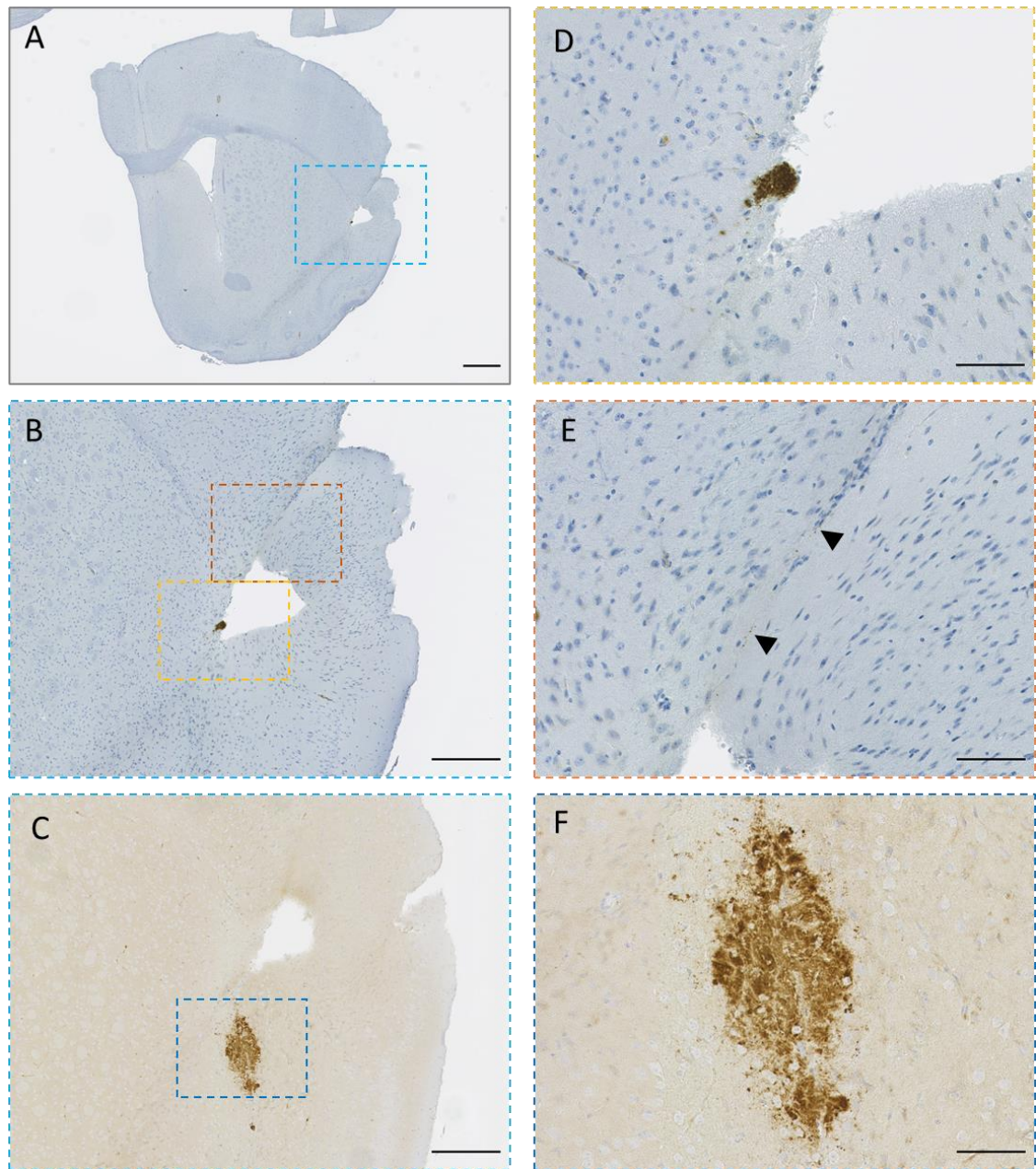


Figure 4-8: PrP^D deposition using 6H4 antibody in the brain of a C57BL/6 mouse (M00111889) inoculated with Nor-R isolate.

Representative images from two experiments show the difference in deposition at two coronal levels. A, B, D, E: A small disorganised plaque/aggregate is present in the right cortex, with fine punctate deposition associated with a needle track (E, arrowheads) C, F: A large disorganised plaque (multicentric) is visible in the right cortex, surrounded by fine punctate deposition. Scale bars: A: 500µm; B, C: 200µm; D, E, F: 50µm.

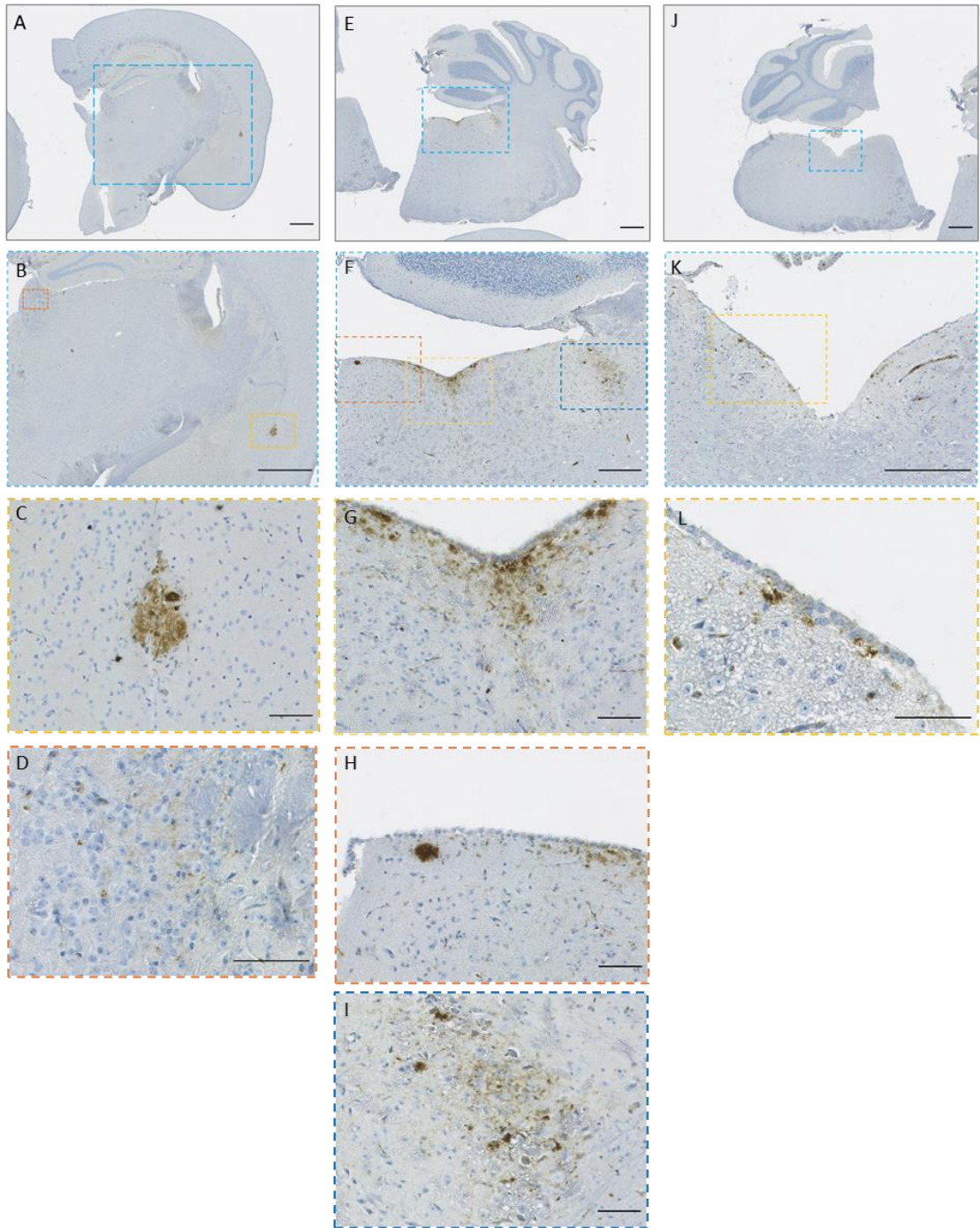


Figure 4-9: PrPD deposition using 6H4 antibody in the brain of a C57BL/6 mouse (M00111925) inoculated with Norwegian reindeer CWD isolate.

Large disorganised plaque present in the right cortex (C), fine punctate and intragial staining in the habenular nucleus of the thalamus (D). Fine punctate to coarse granular staining in the medulla beneath the fourth ventricle with occasional disorganised plaques (G,H,L), similar staining in the locus coeruleus (I). Scale bars; A,E,J,B,F: 500µm; K: 200µm; C,D,G,H,I,L: 50µm

4.3.8 *Alternative staining in CWD inoculated mice*

4.3.8.1 *Haemosiderin*

The analysis of immunohistochemical deposition of PrP^D was complicated by non-specific staining associated with blood vessels, and the occasional identification of a brown pigment which appeared very similar to PrP^D plaques. In one mouse inoculated with the Nor-M isolate, these plaques localised to an area of needle track damage. Similar deposits were also identified in two mice inoculated with the Can-M isolate. This pigment was judged to be haemosiderin due to the shape of the plaques and refractile appearance under light microscopy (Figure 4-10).

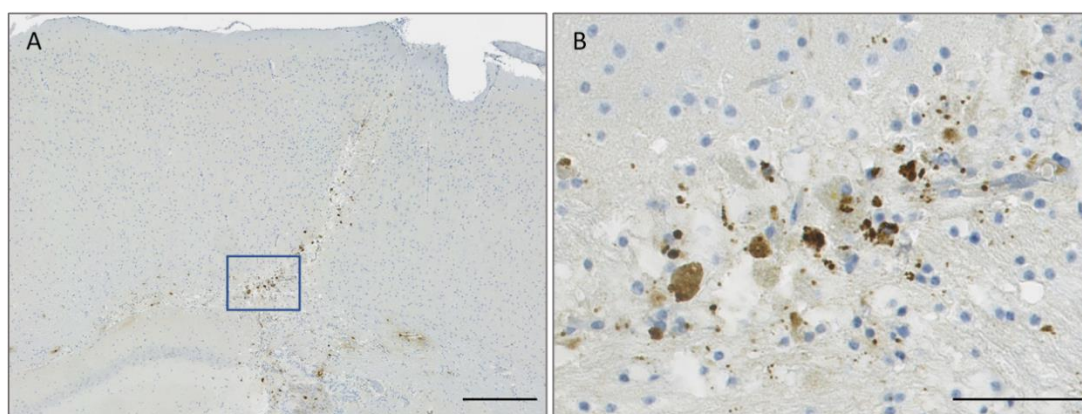


Figure 4-10: Haemosiderin deposition following a needle-track in the brain of a Nor-M mouse found dead at 138dpi.

Haemosiderin deposits were reminiscent of PrP plaques, but distinguished by their rounded to oval shape and slightly refractile appearance under a light microscope.

Scale bars: (A) 200µm (B) 50 µm

4.3.8.2 Unusual staining type

Further complicating the analysis, an unusual staining type was identified in a considerable number of mice regardless of CWD isolate. Staining was specific and not observed in no-primary or isotype controls. Often this staining was weak and non-distinct, disappearing into the neuropil, and affected small focal areas often in the thalamus or striatum. However, in some animals, this non-specific pattern was more intense with punctate pericellular staining (see Figure 4-11B) that appeared similar to disease associated PrP^D.

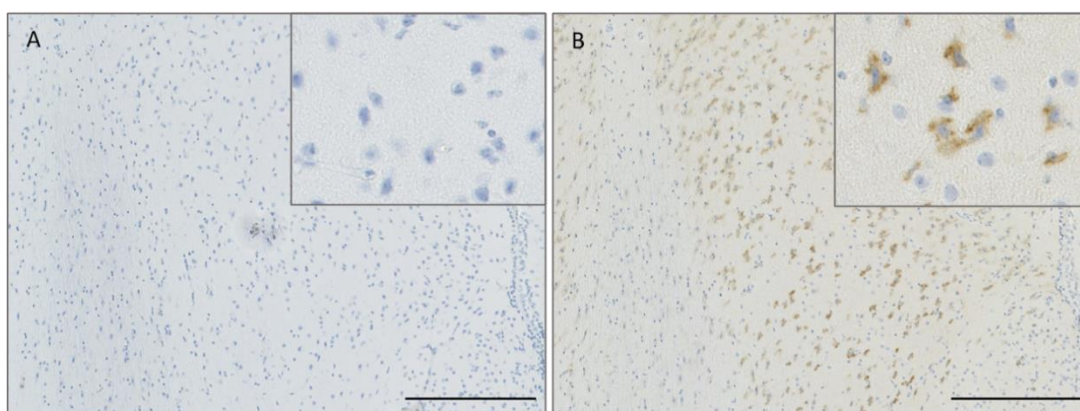


Figure 4-11: Unusual staining observed upon transmission of CWD isolates in wild-type mice.

Representative 'unusual' staining in the striatum of an RIII mouse inoculated with Nor-R isolate. (A) No primary antibody control (B) Primary antibody 6H4. Staining was pericellular and varied from punctate (see inset) to diffuse. In all cases, staining affected focal regions of the brain, affecting the grey matter and most often the striatum and thalamus. Scale bars 200µm

4.3.9 Biochemical characterisation of CWD transmission in the brains of wild-type mice

To screen wild-type mice for the presence of proteinase K (PK) resistant PrP (PrP^{Sc}), western blotting was performed on PK treated brain homogenates. This was undertaken for the three transmissions with limited evidence of transmission based on IHC, as the blotting of half-brain homogenates may allow detection of PrP^{Sc} that was not detected in the specific brain areas analysed by IHC.

4.3.9.1 Optimisation of western blotting

Due to the expected low levels of PrP^{Sc} in these samples, a previously published high-speed centrifugation concentration step was included to increase western blot sensitivity whilst also preserving western blot profile and allowing for processing of large numbers of samples [435]. To optimise and ensure the capture of PrP^{Sc} by this method, preliminary experiments using a positive 10% brain homogenate (scrapie 87V) were undertaken. Following PK digestion, centrifugation at 16100 x g for 1 hour at 4°C allows the separation of sample into pellet (containing insoluble PrP^{Sc}) and supernatant phase. Figure 4-12 shows the efficient recovery of PrP^{Sc} of a control (scrapie 87V) brain using this method with easily detectable PrP^C and PrP^{Sc} in the – and + PK treated pelleted fractions (lanes 5 and 6) respectively.

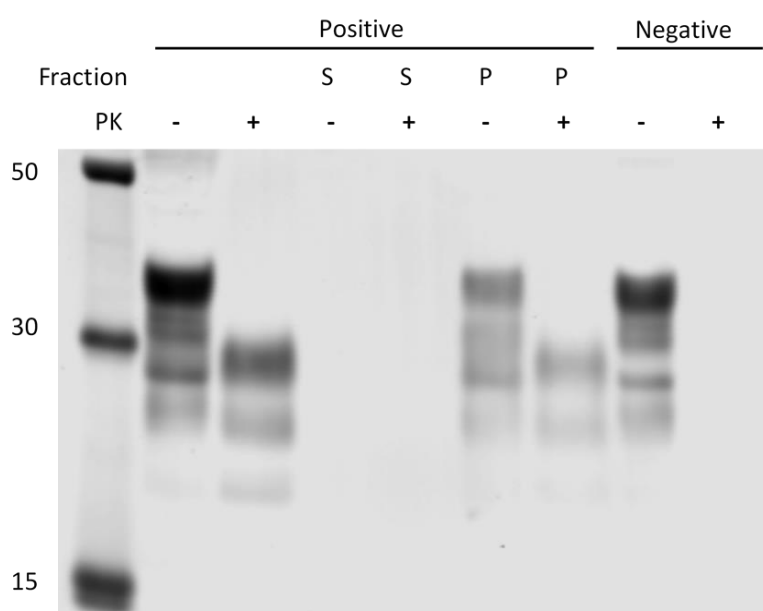


Figure 4-12: Optimisation of western blot protocol. Positive control: 87V scrapie, VM mouse. Negative control: Uninfected RIII mouse. Lane 1 shows untreated positive brain homogenate (10µl), and lane 2 PK-digested positive brain homogenate (10µl treated with 50 µg/µl PK). Duplicate 10µl samples, with and without PK treatment, were diluted 20x with 1X PBS to give a total of 200µl. These samples were centrifuged at 16100 x g for 1 hour to produce supernatant (S) and pellet (P) fractions.

4.3.9.2 Western blotting of Canadian transmissions

Western blot results are summarised in Table 4-4. Western blotting of 43 half-brains from mice inoculated with the Can-M isolate failed to detect PrP^{Sc} in any of these samples. To give a good representation of positivity in the transmission of Can-WT isolate, four mice from each of VM, RIII and C57BL/6 lines with positive IHC results were selected for western blot analysis. Representative western blot results are shown in Figure 4-13. Banding patterns were similar in all three mouse lines, with a predominant diglycosylated band and same size of the unglycosylated fragment (~20-21kDa).

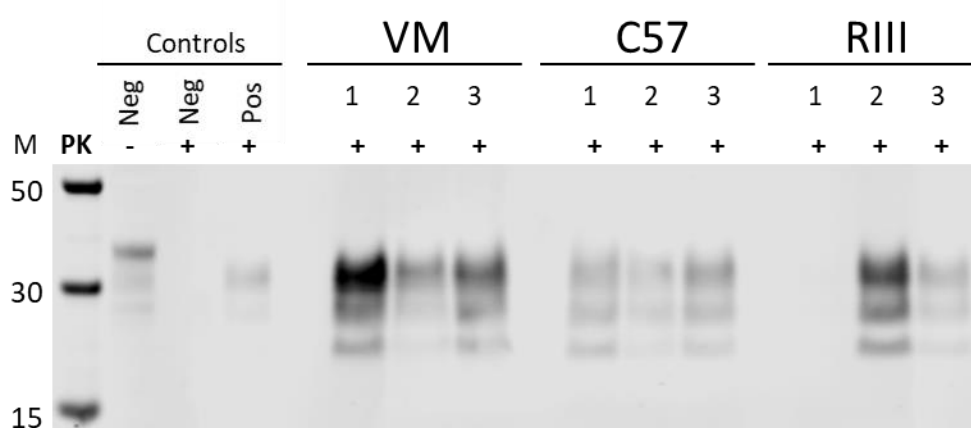


Figure 4-13: Western blot analysis of PrP^{Sc} in the brains of three lines of wild-type mice inoculated with Can-WT isolate.

Membrane was probed with antibody 6H4. Tissue equivalent per lane was: Neg -PK 0.5mg, Neg and Pos +PK 1mg, experimental samples 5mg. Positive control – 87V in a VM mouse, negative control – RIII mouse. Sample RIII (1) was positive but only visible after enhancement/high contrast.

4.3.9.3 Western blotting of Norwegian transmissions

Western blot results are summarised in Table 4-5. A total of 50 brains from mice inoculated with the Norwegian reindeer CWD isolate were screened for PrP^{Sc} by western blotting, with no positives identified. Representative western blotting is shown in Figure 4-14. This included two mice that were positive by IHC, and may be explained by the low levels of PrP^D in these brains outwith the limit of detection of western blotting. A total of 44 brains from mice inoculated with the Norwegian moose CWD isolate were available for testing. As described above,

none of these brains had detectable PrP^D with IHC, however samples from two mice were positive by western blot. One RIII mouse had a clear banding pattern, in which the unglycosylated fragment appeared to have a slightly lower molecular weight (~19kDa) than the 87V scrapie control and Canadian isolates (see Figure 4-14). Interestingly this mouse also displayed clinical signs and vacuolation but was negative for prion protein deposition by IHC. A second C57BL/6 mouse with no clinical or pathological evidence of transmission, also produced a very weak signal on western blot, which was only detectable using image enhancement.

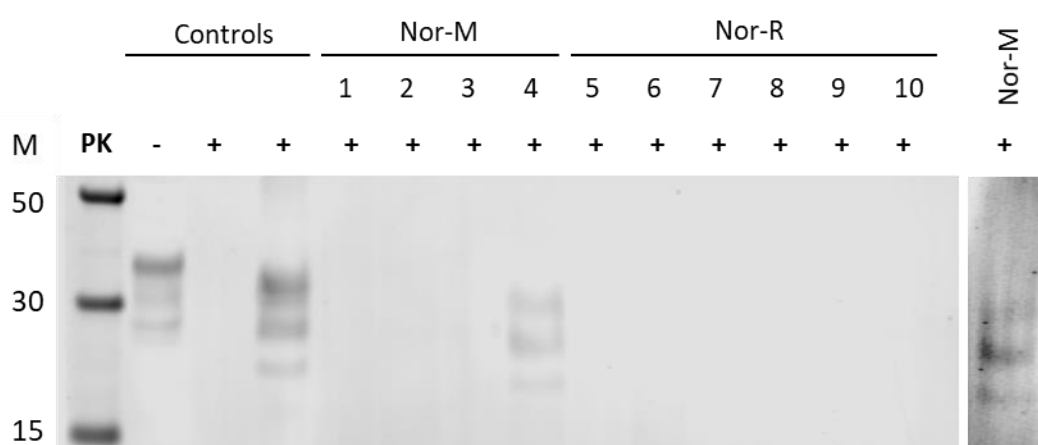


Figure 4-14: Western blot for the detection of PrP^{Sc} in the brains of RIII mice inoculated with (A) Nor-M (lanes 1-4) and Nor-R (lanes 5-10) isolates and (B) a C57/Bl6 mouse inoculated with Nor-M with detectable PrP^{Sc} after image enhancement..

Membranes were probed with antibody 6H4. PrP^{Sc} detected in the brain of a clinically positive, IHC negative RIII mouse (111068), lane 4. Tissue equivalent per lane: Neg -PK 0.5mg, Neg and Pos +PK 1mg, experimental samples 5mg.

4.3.10 Rt-QuIC amplification for the detection of CWD in mouse brain homogenates.

Whilst detection of PrP^{Sc} by immunohistochemistry and western blotting remain the most commonly used methods for demonstration of prion infection, development of assays such as RT-QuIC, allow for the ultra-sensitive detection of amyloid seeding activity which correlates well with prion infectivity by bioassay [92]. I used an RT-QuIC protocol with full-length recombinant bank vole PrP (23-230) as substrate to screen for PrP^{Sc} seeding activity in the brains of wild-type mice inoculated with CWD isolates. Bank vole recombinant PrP has been described as the universal substrate for RT-QuIC [96], as it has been shown to amplify PrP^{Sc} from different species/prion diseases, including Norwegian and North American CWD isolates [95].

Due to the lack of remaining inocula from the mouse transmissions, brain homogenates used in previous PMCA experiments including CWD-positive and uninfected white-tailed deer were used as controls (Table 3-3 and Figure 3-17). Dilutions of CWD-positive brain homogenate from 10^{-2} to 10^{-5} amplified efficiently in bank vole rec-PrP (produced in-house), with lag times of <15hours (results not shown). Negative control dilutions of cervid brain homogenate (10^{-3} and 10^{-4}) showed no evidence of spontaneous aggregation at 60 hours.

Similarly short lag times <15h were achieved when reactions were seeded with mouse brain homogenates from positive (IHC and WB) C57BL/6 mice inoculated with Can-WT isolate. Two samples were chosen to allow a comparison of RT-QuIC amplification between 'strong' positives (Can-WT1) and 'weak' positives (Can-WT2) based on levels of IHC deposition and intensity of western blot signal. The amplification of Can-WT1 was comparable to the cervid CWD positive control with short lag times and both dilutions amplifying efficiently (Figure 4-15). Comparing isolates at the 10^{-3} dilution, the weaker Can-WT2 isolate had an increased lag time and 'stepped' pattern corresponding to individual wells increasing in signal. Interestingly the more concentrated 10^{-2} dilution increased in signal with a lag time comparable to the positive control,

however maximal fluorescence was not reached. This ‘choking’ of the reaction is attributed to an inhibitors that may be present in more concentrated samples. Negative control reactions seeded with uninfected mouse brain homogenate at 10^{-2} to 10^{-4} dilutions showed no spontaneous aggregation until 50 hours. A cut-off time of 45 hours and a threshold of positivity were determined as described in section 4.2.8. Whilst the 10^{-4} dilution of Can-WT2 did show an increase in fluorescence, the lag time of 40-45 hours pushed this close to the non-specific spontaneous conversion seen in unseeded and negative controls.

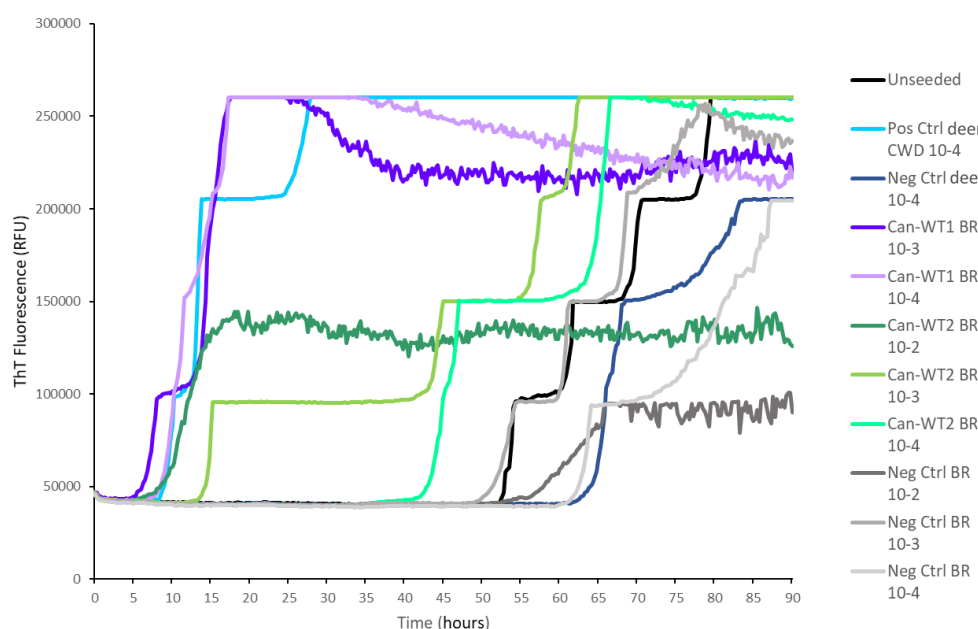


Figure 4-15: RT-QuIC amplification of CWD using bank vole recombinant substrate.

Positive and negative WTDbrain homogenates are used as controls. RT-QuIC reactions were seeded with tenfold serial dilutions ($10^{-2,3,4}$) of brain homogenates from two mice inoculated with Can-WT isolate, either strongly (Can-WT1) or weakly positive (Can-WT2) based on IHC deposition and WB signal strength. Unseeded reactions and uninfected mouse brain homogenates are also included as negative controls. Reactions are shown to 90h to demonstrate the spontaneous conversion seen after 45 hours. ThT fluorescent signal is shown as an average of 4 replicate wells against time.

Nine mouse brain homogenates from transmission experiments with Norwegian isolates (Nor-R and Nor-M) were tested using bank vole rec-PrP RT-QuIC. Two Can-WT samples were included for comparison, and both amplified efficiently (Figure 4-16A). Two mice inoculated with Norwegian moose CWD found to be negative by IHC, but positive by western blot (Nor-M1, Nor-M2) also amplified efficiently, with a lag time of only 5 hours, compared to 10 hours for the Canadian isolate (Figure 4-16B). This was unexpected due to the presumed low levels of PrP^{Sc} in the sample, particularly in Nor-M1, which was only just detectable on western blot. Two mice inoculated with the Norwegian reindeer CWD isolate that were positive by IHC, but negative on western blotting, were also positive by RT-QuIC (Figure 4-16C). The amplification was comparatively weak and Nor-R1 at 10⁻² dilution exhibited a similar 'choking' of the reaction seen with the 'weak' Canadian sample. The 10⁻³ dilution was positive with a longer lag time of >20 hours, and 3 of 4 wells positive by 45 hours. Likewise, the Nor-R2 at 10⁻² dilution exhibited choking of the reaction with 4 of 4 wells positive but not reaching maximal fluorescence by 45 hours. At 10⁻³ there was no increase in fluorescence, which may be expected due to the comparative lack of IHC deposition seen in Nor-R2 (Figure 4-8) to Nor-R1 (Figure 4-9).

Five brain homogenates from mice inoculated with Norwegian isolates and negative by previous analyses were also tested, as outlined in Table 4-8, and shown in Figure 4-16D. These samples were negative with the exception of Nor-R3, which exhibited amplification in 2 of 4 wells after 25 hours. It is unclear whether this represents positivity in a sample that was undetectable by less sensitive methods such as IHC and western blot. Alternatively, this amplification may be non-specific due either to random debris (providing a nucleus around which spontaneous conversion can occur), or may be due to the position of these wells on the outer edge of the plate where the kinetics may promote spontaneous conversion. A sample identified as having high levels of unusual staining on IHC (Figure 4-11) was included (Nor-R4) and did not show any evidence of positivity, suggesting that this unusual staining type is not prion-disease related.

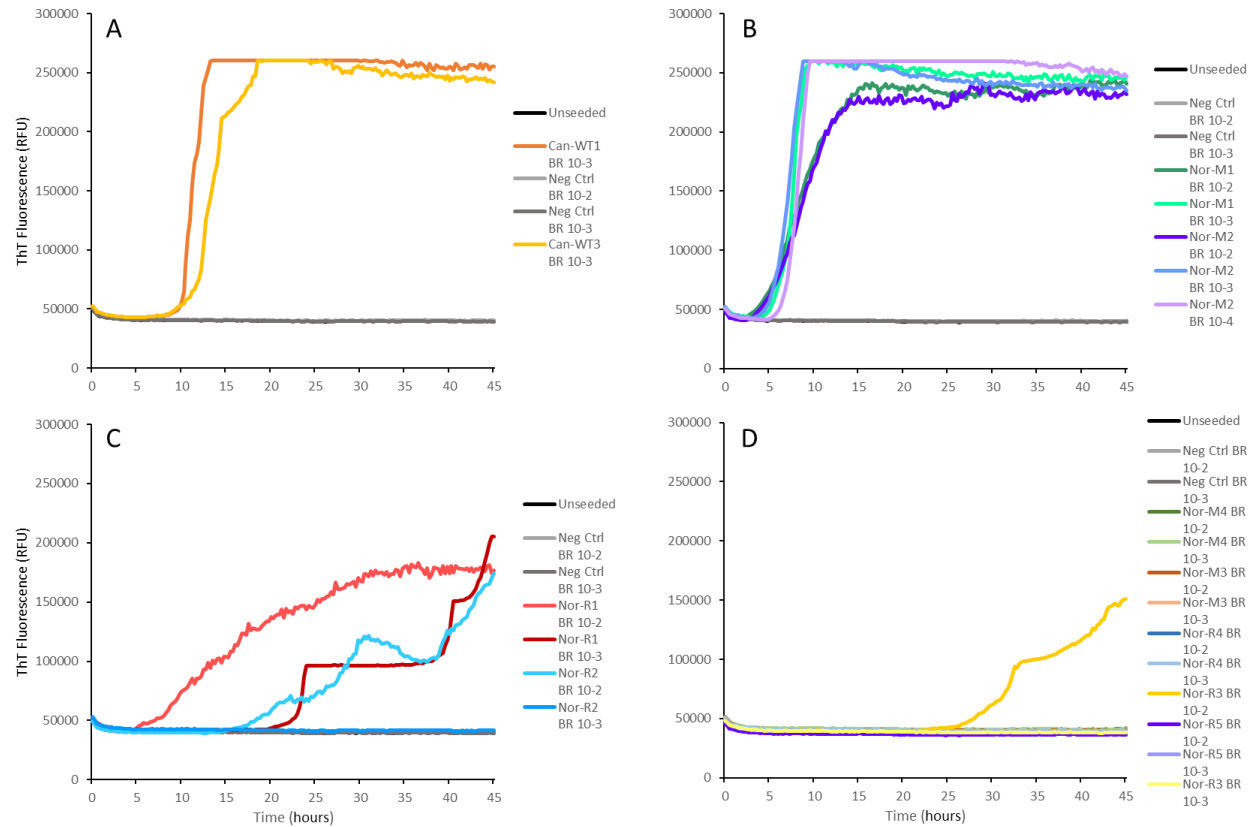


Figure 4-16: Amplification of a panel of mouse brain homogenates from Nor-M, Nor-R and Can-WT transmissions. Reactions were seeded with: (A) brain homogenates (10^{-3} dilution) from two mice (Can-WT1, Can-WT3) with positive IHC and WB results (B) serial dilutions of brain homogenates 10^{-2} - 10^{-4} from one mouse (Nor-M1) and 10^{-2} and 10^{-3} dilutions from a second mouse (Nor-M2) with positive WB results (C) 10^{-2} and 10^{-3} dilutions of brain homogenates from two mice (Nor-R1 and Nor-R2) with positive IHC results (D) 10^{-2} and 10^{-3} dilutions of brain homogenates from five mice (Nor-M3, Nor-M4, Nor-R3, Nor-R4, Nor-R5) with negative IHC and WB results. ThT fluorescence signal is shown as an average of 4 replicate wells against time.

4.3.11 *Is Lymphoid replication an important feature of primary passage of CWD in wild-type mice?*

Published mouse transmission experiments variably include analysis of lymphoid tissues, however there is evidence that lymphoid replication of prions plays an important role in cross-species transmission and adaptation [108]. CWD in North American cervids and Norwegian reindeer are lymphotropic in their natural hosts, whilst CWD in Norwegian moose does not appear to be. Transmission experiments provide an opportunity to determine whether this characteristic is maintained following transmission to mice, and if lymphoid replication is an important feature of CWD cross-species transmission.

Table 4-8: RT-QuIC analysis of mouse brain homogenates from Can-EM, Nor-R and Nor-M transmissions.

Inoculum	Thesis ID	Mouse number	Mouse line	Seed dilution	Result ¹	
Can-WT	Can-WT1	M00132818	C57BL/6	10 ⁻³	+	12/12
				10 ⁻⁴	+	4/4
Can-WT	Can-WT2	M00132842	C57BL/6	10 ⁻²	+	8/8
				10 ⁻³	+	6/8
				10 ⁻⁴	-	1/4
Can-WT	Can-WT3	M00130873	VM	10 ⁻³	+	4/4
Nor-M	Nor-M1	M00111904	C57BL/6	10 ⁻²	+	4/4
				10 ⁻³	+	4/4
Nor-M	Nor-M2	M00111068	RIII	10 ⁻²	+	4/4
				10 ⁻³	+	4/4
				10 ⁻⁴	+	4/4
Nor-M	Nor-M3	M00112014	RIII	10 ⁻²	-	0/4
				10 ⁻³	-	0/4
Nor-M	Nor-M4	M00110751	VM	10 ⁻²	-	0/4
				10 ⁻³	-	0/4
Nor-R	Nor-R1	M00111925	C57BL/6	10 ⁻²	+	4/4
				10 ⁻³	+	3/4
Nor-R	Nor-R2	M00111889	C57BL/6	10 ⁻²	+	4/4
				10 ⁻³	-	0/4
Nor-R	Nor-R3	M00111907	C57BL/6	10 ⁻²	B	2/4
				10 ⁻³	-	0/4
Nor-R	Nor-R4	M00106969	RIII	10 ⁻²	-	0/4
				10 ⁻³	-	0/4
Nor-R	Nor-R5	M00109296	VM	10 ⁻²	-	0/4
				10 ⁻³	-	0/4

¹ Results are given as positive '+', negative '-' or borderline 'B', followed by the number of wells reaching threshold before the prescribed cut off/total number of wells

4.3.11.1 Immunohistochemical detection of PrP^D in the spleens of wild-type mice inoculated with CWD.

Spleen sections from CWD inoculated mice were stained using ROS-BH1 clone antibody [430] and standard protocol (see 4.2.5). Spleens were scored based on the number of germinal centres/B cell follicles with positive labelling, and the intensity of staining (see Figure 4-17). Results in full are listed in the appendix (Table 7-1). A total of eighteen mice inoculated with Nor-R showed positive labelling in the spleen that displayed the expected pattern for PrP^D deposition. However, of these 18, only four were scored as having strongly positive staining, which was generally identified in a higher number of follicles than the other 14 mice which had very weak staining, often in only one or two follicles (Figure 4-17). Six mice inoculated with the Nor-M isolate were scored positive, with only one mouse showing strong staining. A panel of 28 spleens (including 4-5 mice from each of VM, RIII and C57BL/6) from the Canadian transmissions were also tested, the majority of which were negative, with only 2 RIII mice scoring weakly positive in 1-2 follicles from the Can-WT inoculated group.

IHC results in the spleen did not correlate well with staining in the brain; neither of the two Nor-R inoculated mice with positive PrP^D in the brain were positive in the spleen. This was also the case in transmissions of Canadian CWD isolates, as the majority of mice from the Can-WT transmission were positive for PrP^D in the brain but were not IHC positive in the spleen. It was therefore important to consider whether the staining may be residual PrP^C which was not fully denatured during the fixation and pre-treatment of tissues. Cellular PrP^C is removed by PK digestion, which may be achieved by paraffin embedded tissue (PET) blotting of spleen sections, or western blotting of spleen homogenates. The latter was undertaken due to the lack of available controls for comparison in PET blotting experiments, and difficulty in performing this method on formic-treated tissues.

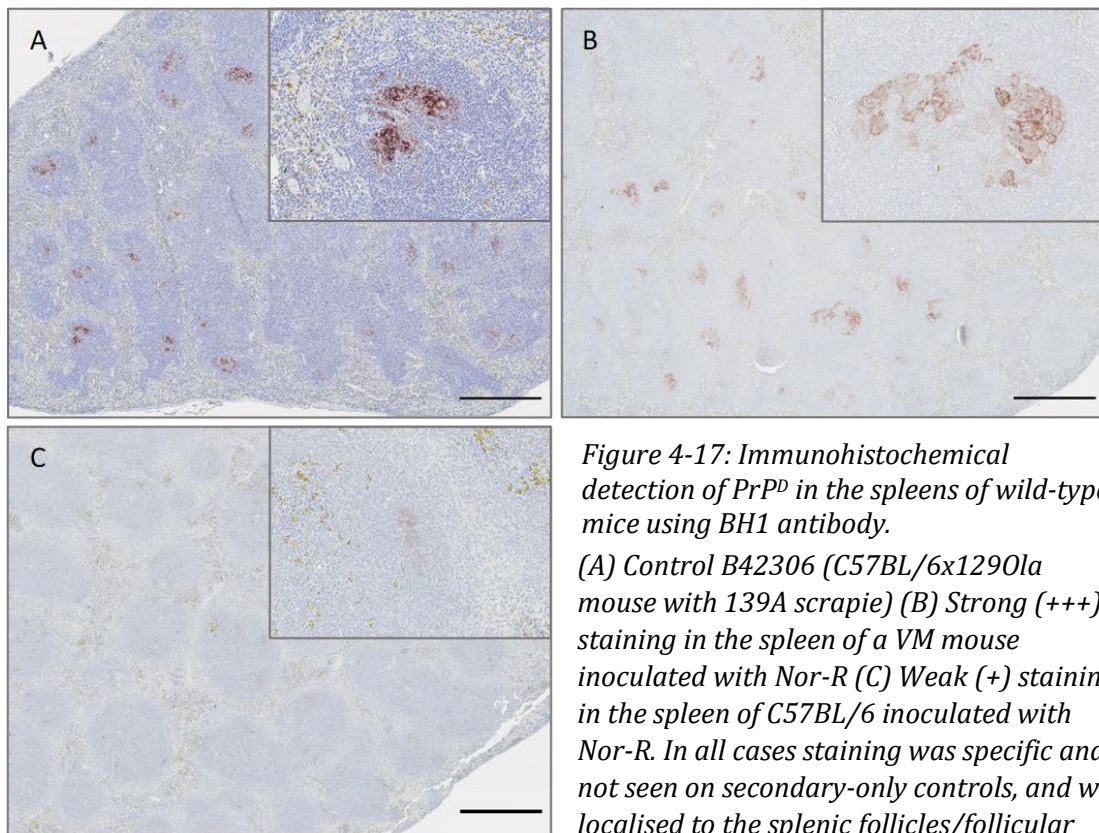


Figure 4-17: Immunohistochemical detection of PrP^D in the spleens of wild-type mice using BH1 antibody.

(A) Control B42306 (C57BL/6x1290la mouse with 139A scrapie) (B) Strong (+++) staining in the spleen of a VM mouse inoculated with Nor-R (C) Weak (+) staining in the spleen of C57BL/6 inoculated with Nor-R. In all cases staining was specific and not seen on secondary-only controls, and was localised to the splenic follicles/follicular dendritic cells. Scale bars 500μm

4.3.11.2 NaPTA precipitation of PrP^{Sc} from wild-type mice spleens

Sodium phosphotungstic acid (NaPTA) precipitation has previously been shown to selectively capture and concentrate PrP^{Sc} increasing the sensitivity of detection upon immunoblotting [159, 431, 432]. For initial optimisation, both 'long' and 'short' NaPTA protocols (section 4.2.7.3) and a PK-treated non-NaPTA precipitated spleen sample subjected to high-speed centrifugation were included for comparison. Samples prepared using all three protocols gave strong signals on western blot, with little difference between 'long' and 'short' NaPTA protocols (Figure 4-18). The non-NaPTA precipitated sample shows a slight smearing effect above the banding pattern, and a slight blurring of the bands, but less than would be expected from a homogenate from a highly concentrated spleen sample. However, the 'short' protocol was used for further experiments, as it combined time-efficient sample preparation and the best resolution on blots.

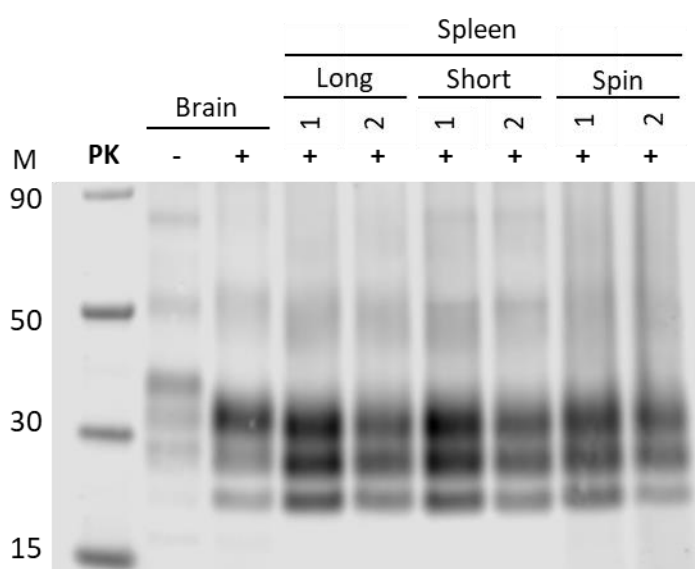


Figure 4-18: Optimisation of NaPTA protocol for precipitation of PrP^{Sc} in wild-type mouse spleens.

Scrapie positive (87V) mouse brain was included as a western blot and PK control. Scrapie positive (ME7 strain) C57BL/6 mouse spleens (1 and 2) were used for optimisation. 'Long' NaPTA protocol features an overnight incubation, whereas 'short' protocol incubates for 30 minutes. 'Spin' protocol does not use NaPTA precipitation but high speed centrifugation (15800 x g) for 1 hour. Equivalent tissue weights loaded: brain minus PK 0.2mg, plus PK 1mg, spleens 5mg.

To test whether immunolabelling of PrP^D in spleens from mice inoculated with Norwegian CWD correlated with the presence of PrP^{Sc}, spleens from three mice with high IHC scores were selected for NaPTA precipitation and western blot. A fourth mouse inoculated with Can-WT CWD was included for comparison. In the positive control sample (spleen from ME7-infected mouse), PK-resistant PrP^{Sc} was evident on the blot. The experimental spleen samples were all negative for PrP^{Sc}, although a weak non-specific band was visualised in some test samples and the negative control (Figure 4-19). A repeat of NaPTA precipitation and western blotting reproduced these results (not shown). Therefore it was judged that the IHC labelling may either be non-specific, due to PrP that is PK sensitive (PrP^C or senPrP^{Sc}), or that PrP^{Sc} concentrations in the spleen are below the threshold of detection for NaPTA precipitation and western blotting. To determine if the latter was true, spleen samples were assayed using RT-QuIC.

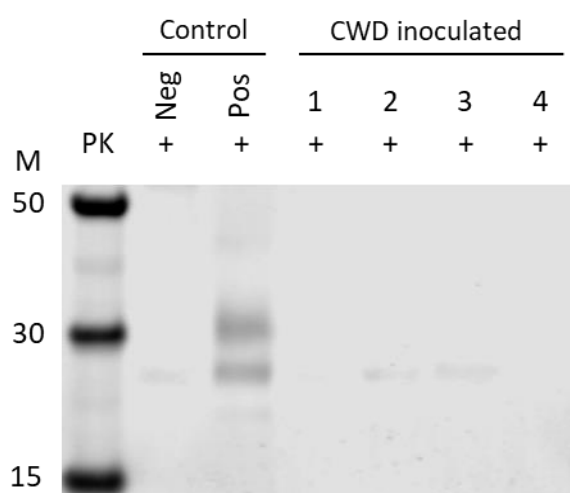


Figure 4-19: NaPTA precipitation and western blotting of wild-type mouse spleens.

Positive control: Me7 positive spleen sample. Negative control: Negative RIII spleen sample. Experimental CWD inoculated spleens as follows: (1) Nor-R7 (2) Nor-M5 (3) Nor-R3 (4) Can-WT1 (see Table 4-9). Equivalent tissue weight: 5mg.

4.3.11.3 RT-QuIC amplification of spleen samples from wild-type mice

In order to establish a protocol for testing spleen samples, I began by comparing the amplification of a known western-blot positive spleen sample with and without NaPTA pre-treatment. NaPTA pre-treatment has been shown to improved detection of CWD seeding activity in a variety of samples including lymphoid tissues [300, 436], whole blood [437], and faeces [438]. Comparison of the positive control spleen sample from a scrapie (ME7) infected mouse before and after NaPTA precipitation showed a cleaner result by western blot (Figure 4-18), and it was hypothesised that NaPTA precipitation would be an

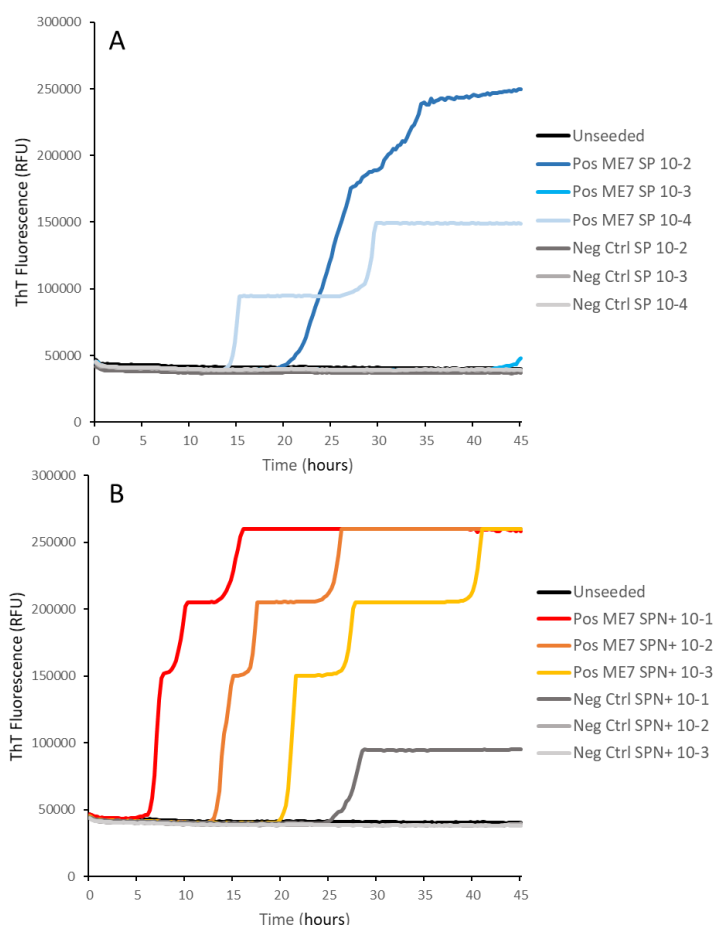


Figure 4-20: RT-QuIC amplification spleen samples before (A) and after NaPTA precipitation (B).

Positive spleen material was provided by pooling spleen homogenates from two ME7 infected mice, which had previously been confirmed as positive by western blotting. Negative spleen material was provided from a non-infected RIII mouse, confirmed negative by western blotting. Dilutions were prepared from either 10% spleen homogenates (SP) or from NaPTA precipitated spleen homogenates in 0.1% sarcosyl/PBS (SPN+). Unseeded control reactions showed no increase in fluorescence (spontaneous conversion) at 45h.

effective pre-treatment for mouse spleen samples in RT-QuIC, due to the removal of inhibitory substances such as blood.

Untreated spleen samples (Figure 4-20A) showed inconsistent amplification, with the 10^{-2} dilution increasing after a lag phase of 20 hours, the 10^{-3} dilution failing to amplify in any wells, and the 10^{-4} dilution showing the shortest lag time at 15 hours, but with only 2/4 wells positive at the end of the run. The NaPTA pre-treated samples amplified very well, with the 10^{-1} dilution becoming positive at only just over 5 hours and 4/4 wells positive. A consistent dilution series was seen in the 10^{-2} and 10^{-3} samples, with an increased lag time of approximately 5 hours for each subsequent dilution. In comparison with the non-treated spleen samples the fluorescence traces were clean with 'steps' consistent with each well becoming positive. The negative control spleen showed some non-specific amplification after 25 hours in a single well, which may have been due to NaPTA precipitate not being fully solubilised or debris providing a nucleus for amyloid formation.

RT-QuIC analysis was then performed on the spleen samples previously identified as having strong staining on IHC (Figure 4-17) but testing negative by NaPTA precipitation and western blotting (Figure 4-19). NaPTA precipitated spleen homogenates at 10^{-1} to 10^{-3} dilutions were used to seed RT-QuIC reactions. Reactions seeded with positive control spleens produced results comparable to the previous experiment with a reduced amplification at the 10^{-3} dilution, which may be due to sample variability. Negative control spleen samples showed minimal amplification with only one well becoming positive between 40 and 45h at the 10^{-2} dilution (Figure 4-21A). The Can-WT1 spleen homogenate was strongly positive with convincing amplification at <10hours in all three dilutions. The spleens from mice inoculated with Norwegian CWD isolates showed comparatively little seeding activity, with only 2 wells showing increased fluorescence after a lag time of greater than 30 hours in reactions seeded with Nor-M5 at 10^{-2} and Nor-R7 at 10^{-1} . These samples were classed as borderline owing to the low confidence of their positivity due to the very prolonged lag time of >30 hours and that only 2 of 4 wells showing any amyloid

formation/increase in fluorescence. These inconclusive results and the completely negative results from Nor-R3 (Figure 4-19D), make it difficult to argue that the strong staining seen on IHC of these spleens is disease-specific.

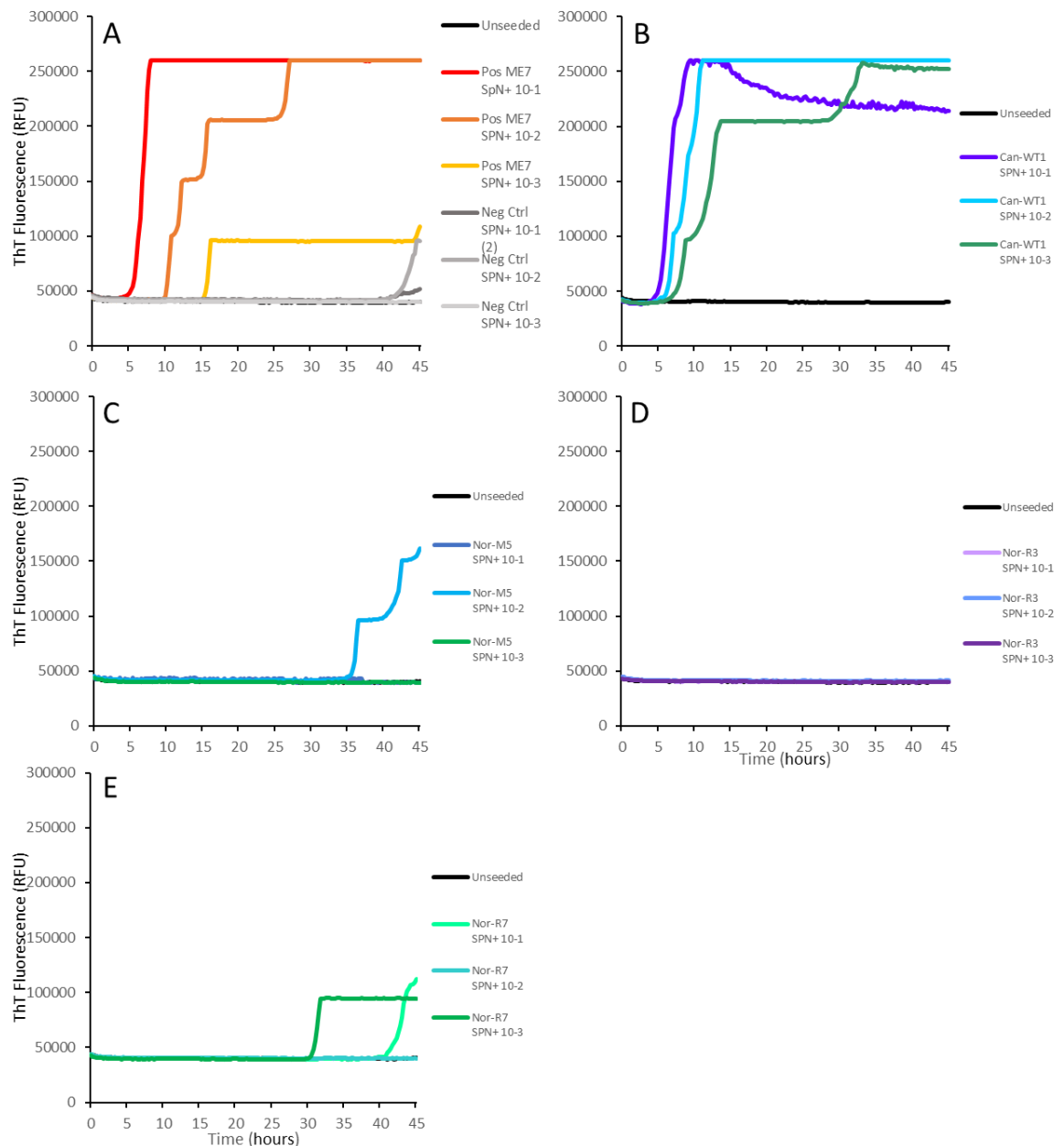


Figure 4-21: RT-QuIC optimisation and testing using NaPTA precipitated spleen samples.

(A) Positive and negative controls (B) Can-WT1 (C) Nor-M5 (D) Nor-R3 (E) Nor-R7. Positive control spleen material was a pooled homogenate from two ME7 positive mice. Positive and negative control samples were confirmed by western blotting. Reactions were seeded with serial dilutions ($10^{-1,2,3}$) of NaPTA precipitated spleen samples (SpN+) solubilised in 0.1% sarcosyl/PBS, diluted in PBS. Unseeded control reactions showed no increase in fluorescence (spontaneous conversion) at 50h.

To continue this investigation further, additional spleens from mice inoculated with Norwegian isolates were tested, including mice with brains that had tested positive by RT-QuIC (see Table 4-9). No amyloid seeding activity was detected in any of the samples (0/8) (Figure 4-22A,B). A repeat of the previously borderline positive Nor-M5 sample was negative, with only one well becoming positive at over 40 hours in line with the spontaneous conversion seen in the unseeded control. A panel of 6 spleens from each of the Canadian transmissions was also tested. Spleens from Can-WT inoculated mice were consistently positive (6/6) (Figure 4-22C) with short lag times between 5 and 10 hours. Interestingly, whilst five of the six Can-M inoculated mice did not show any

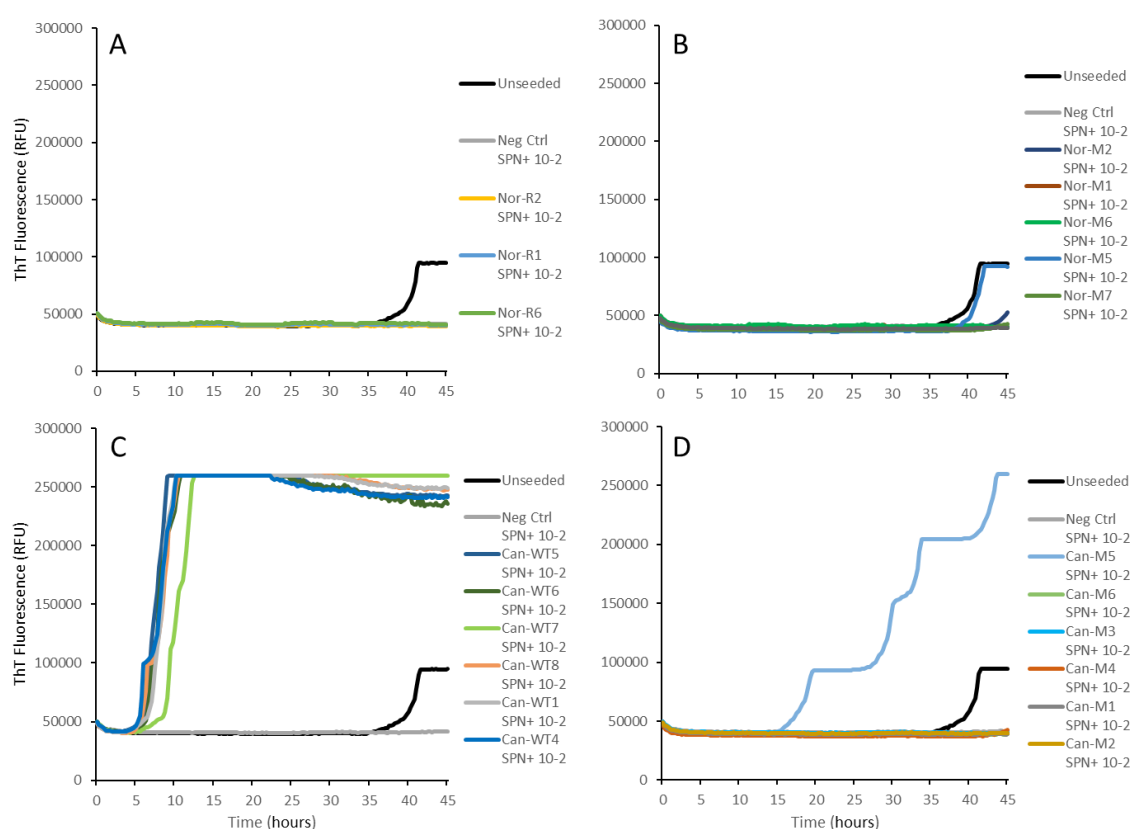


Figure 4-22: RT-QuIC amplification of NaPTA precipitated spleen samples.

Spleen material from transmissions Nor-R (A), Nor-M (B), Can-WT (C) Can-M (D) was NaPTA precipitated and pellets resuspended in 0.1% sarcosyl/PBS (SPN+). NaPTA product was used to seed RT-QuIC reactions at a dilution of 10⁻². Negative spleen material from a non-infected control animal showed no increase in fluorescence at 45h, and unseeded reactions showed an increase in one well at 40h (artefact).

seeding activity in spleen samples, one (Can-M5) showed convincing amplification with 4/4 wells positive albeit after an extended lag period of 15 hours.

Table 4-9: RT-QulC analysis of NaPTA precipitated spleen samples from CWD transmissions in wild-type mice.

Inoculum	Thesis ID	Mouse number	Mouse line	Seed dilution	Result ¹	
Can-WT	Can-WT1	M00132818	C57BL/6	10 ⁻¹	+	4/4
				10 ⁻²	+	8/8
				10 ⁻³	+	4/4
	Can-WT4	M00132826	C57BL/6	10 ⁻²	+	4/4
	Can-WT5	M00131453	RIII	10 ⁻²	+	4/4
	Can-WT6	M00131467	RIII	10 ⁻²	+	4/4
	Can-WT7	M00130315	VM	10 ⁻²	+	4/4
	Can-WT8	M00130863	VM	10 ⁻²	+	4/4
Can-M	Can-M1	M00132819	C57BL/6	10 ⁻²	-	0/4
	Can-M2	M00132828	C57BL/6	10 ⁻²	-	0/4
	Can-M3	M00130300	RIII	10 ⁻²	-	0/4
	Can-M4	M00132481	RIII	10 ⁻²	-	0/4
	Can-M5	M00130307	VM	10 ⁻²	+	4/4
	Can-M6	M00130525	VM	10 ⁻²	-	0/4
Nor-M	Nor-M5	M00111901	C57BL/6	10 ⁻¹	-	0/4
				10 ⁻²	B	3/8
				10 ⁻³	-	0/4
	Nor-M1	M00111904	C57BL/6	10 ⁻²	-	0/4
	Nor-M6	M00111903	C57BL/6	10 ⁻²	-	0/4
	Nor-M2	M00111068	RIII	10 ⁻²	-	0/4
	Nor-M7	M00112507	RIII	10 ⁻²	-	0/4
	Nor-M8	M00110628	VM	10 ⁻²	-	0/4
Nor-R	Nor-R3	M00111907	C57BL/6	10 ⁻¹	-	0/4
				10 ⁻²	-	0/4
				10 ⁻³	-	0/4
	Nor-R2	M00111889	C57BL/6	10 ⁻²	-	0/4
	Nor-R1	M00111925	C57BL/6	10 ⁻²	-	0/4
	Nor-R6	M00112274	RIII	10 ⁻²	-	0/4
	Nor-R7	M00109504	VM	10 ⁻¹	B	2/4
				10 ⁻²	-	0/4
				10 ⁻³	-	1/4

¹Results are given as positive '+', negative '-' or borderline 'B', followed by the number of wells reaching threshold before the prescribed cut off/total number of wells

4.4 Discussion

The passage of prion isolates in experimental animal models (usually laboratory rodents) remains the gold-standard method of strain typing for novel prion diseases. Due to early reports of inefficient transmission of CWD to wild-type mice [315], most of the North American CWD strain typing investigations have been carried out in transgenic mouse models. However the inter-lab variability in transgenic models and scoring criteria can make comparison of results very difficult. The advantages of using a well-defined strain typing panel of wild-type mice include reduced variability and the ability to compare to existing TSE strains, such as the wild-type mouse CWD 409V strain isolated from North American transmission experiments (Diack, pers. comm., [424]).

Whilst transmission rates for the Norwegian (Nor-R and Nor-R) and Can-M isolates were very low, as may be attributed to a strong species barrier between deer and mice, transmission rates were much higher for the Can-WT isolate. One possible explanation for this is the difference in CWD strains in these samples. The experimental white-tailed deer H95/S96 CWD isolate (Can-WT) provided has previously been reported to contain a mixture of Wisc-1 and H95+ strains, which are differentially isolated in tg33 (wt) and tg60 (S96) mice expressing deer PrP [318]. This isolate has also been reported to transmit with efficiency in C57BL/6 mice, producing clinical disease in 7/7 C57BL/6 mice inoculated, whereas isolates containing only the Wisc-1 strain failed to transmit [319]. This is supported by our results, where this isolate transmitted efficiently in C57BL/6 mice and produced clinical disease in 7/17 mice, and pathological evidence of disease in 16/17 mice. We also observed significant transmission of the Can-WT isolate to VM and RIII mice, indicating that this strain can replicate in both *Prnp-a* and *Prnp-b* genotype mice. These results show that the assumed inefficiency of CWD transmission in wild-type mice is not always the case and may vary greatly depending on strain.

Whilst less is known about the hunter-harvested mule deer isolate (Can-M), the lack of transmission in wild-type mice observed may be rationalised if we

consider that this isolate may have contained a Wisc-1 or CWD-1 like prion strain. C57BL/6 mice inoculated with Wisc-1 or Elk CWD had no signs of prion disease (based on clinical signs and western blotting) at over 700 days post-inoculation [319]. There is increasing evidence to suggest that the CWD strains present in Norwegian reindeer and moose are different from those in North America, and different from each other [17, 343, 346]. In bank voles, primary transmissions of Canadian CWD isolates were characterised by high attack rates and relatively short survival times compared to Norwegian moose isolates, which had higher attack rates but longer survival times, and Norwegian reindeer isolates which were barely transmissible. These less efficient transmissions did not appear to be the result of low levels of PrP^{Sc} in the brain homogenates as determined by western blot. Secondary passage of both reindeer and moose isolates in bank voles produced shortened incubation periods and higher attack rates, consistent with adaptation to a new host [346]. The low numbers of transmission seen in our transmission to wild-type mice are similar to these findings, and we may expect to see similar adaptation upon secondary passage in wild-type mice.

Whilst the difference in transmission efficiency we observed between CWD isolates may be due to the CWD strains present in each inoculum, another important possible explanation may be the infectious titre of the CWD inocula. The best estimation of titre is produced by endpoint titration (inoculation of serial dilutions) of the isolate in a common susceptible recipient. Unfortunately, this was beyond the scope of this experiment, and alternative methods such as comparative western blotting or RT-QuIC dilution series were not undertaken due to lack of available material. However, evidence from the published literature provides some evidence to the titre in our experimental inocula. The Can-WT isolate was an experimental isolate produced by passage of CWD in white-tailed deer of the H95/S96 genotype [234] which has been shown to contain high titres of CWD prions upon passage to transgenic cervidised mice, wild-type C57BL/6 mice and hamsters [318, 319]. Less is known about the hunter-harvested mule deer CWD isolate (Can-M), however it has transmitted

with low penetrance in hamsters, and transmission into Tg33 (wt cervid PrP) mice produced incubation periods of ~330 days, suggestive of a low titre (Debbie McKenzie, pers comm). The Norwegian isolates are likewise from natural cases of CWD and may therefore have more variable infectivity titres and levels of PrP^{Sc} [17, 343]. The reindeer brain tissue provided was from the cortex, which was tested by ELISA alongside other brain regions, all of which were strongly positive [17]. In Norwegian moose, labelling was observed in all parts of the brain except the cerebellum but with a more restricted, predominantly intraneuronal deposition pattern [343]. Wadsworth *et al* [439] reported western blotting results on samples from the same animals (for transmission into transgenic mice expressing human PrP) and found that PrP^{Sc} could be readily detected in the reindeer brain homogenate, whereas the moose had lower relative levels of PrP^{Sc} that required NaPTA precipitation for reliable detection [439], suggesting a lower titre in this sample that may be reflected in our own.

Due to the unknown clinical phenotype of Norwegian CWD in wild-type mice, our clinical parameters had to be more inclusive of a variety of signs, which inevitably leads to some clinical suspects being false positives, for example changes to gait due to ageing. However, convincing clinical signs were seen upon transmission of the Can-WT isolate. Clinical signs including ataxia, kyphosis, torticollis, were consistent with previous reports of CWD transmission in rodent models, however dermatitis which has been previously noted [319, 440] was not identified. The incubation periods/survival times we observed were broadly similar to those previously reported for primary CWD transmissions in wild-type mice ([253, 319], Diack, pers. comm.).

Likewise, the prion protein deposition is similar to that described for CWD transmission in various experimental models. We observed that plaque formation was a significant finding, and that this varied between C57BL/6 and RIII (*Prnp-a*) and VM (*Prnp-b*) mice. The frequent perivascular plaques identified in VM mice inoculated with Can-WT isolate are reminiscent of those seen in VM mice inoculated with a mule deer isolate (CWD^{MD1}) from the USA,

further passage of which produced the mouse-adapted plaque forming CWD strain 409V (Diack, pers. comm.[424]). Other USA isolates from mule and white-tailed deer targeted the cochlear nucleus, which was a frequent finding in this study for all three mouse lines inoculated with Can-WT isolate. Experimental transmission of CWD isolates from Korean elk to VM mice produced focal accumulations of PrP^D in the corpus callosum, and perivascular plaques were not a significant finding [253]. Both studies observed low levels of vacuolation, similar to these transmissions. Unfortunately no neuropathology was published for the previous transmission of H95+ CWD in C57BL/6 mice for comparison [319].

Plaque formation is a feature of CWD pathology both in the natural hosts [220, 222] and in experimental small rodent models [149, 268, 346, 440–442]. The reason for frequent plaque formation in CWD affected animals is unknown, however recent research suggests that post-translational modification and shedding of PrP^C are likely to be key factors. PrP^C is tethered to the outer leaflet of the plasma cell membrane by a GPI anchor, and a proportion (10-15%) constitutively shed by the action of a metalloproteinase known as ADAM10. Using an antibody specific to ADAM10 cleaved PrP, Aguilar-Calvo *et al* have shown that plaques in mouse-adapted CWD are largely composed of ADAM10 cleaved PrP lacking the GPI anchor [443]. Lack of membrane anchoring has also been associated with fibrillar plaque formation with a vascular tropism [444–447]. The plaque forming activity of these prions may be association with interaction with heparan sulphate as an endogenous co-factor in the extracellular matrix. GPI anchorless prions consistently bound high levels of heparan sulphate and formed fibrils, and shortening of heparan sulphate chains in transgenic mice prolonged survival and reduced formation of parenchymal plaques [443]. Likewise, the addition of an additional N-linked glycan to produce tri-glycosylated PrP in transgenic mice reduced the interaction with heparan sulphate and changed the neuropathological phenotype of mouse-adapted CWD from plaque forming to fine, granular deposits with a decrease in survival time[448, 449].

The low numbers of mice with evidence of infection when inoculated with Norwegian CWD isolates, and the lack of consistency in detection using different methods (IHC, WB, RT-QuIC) may be explained by the low levels of CWD replication and PrP^{Sc} deposition in these mice. Whilst Nor-R mice showed no clinical signs and no vacuolation, the presence of PrP deposition in two mice could indicate a sub-clinical disease with limited accumulation of PrP^{Sc}. This is consistent with the results of western blotting (which failed to detect PrP^{Sc}) and RT-QuIC (which gave positive results) in brain samples from these mice, suggesting that PrP^{Sc} levels are below the limit of detection of western blot. The identification of PrP^{Sc} by western blot in two mice inoculated with Norwegian moose CWD that showed no PrP^D deposition by immunohistochemistry, is more difficult to explain. One possibility is that the extent of PrP^D deposition was very limited and restricted to areas of the brain that were not sampled for IHC during this study. The western blot signal was stronger in one mouse that also displayed clinical signs and low levels of TSE vacuolation, whereas the second mouse was clinically and pathologically negative, and the signal on western blot could only be detected after image enhancement. Despite this, both samples amplified very efficiently in bank vole substrate by RT-QuIC. In contrast, RT-QuIC reactions seeded with brain homogenates from IHC-positive Nor-R mice showed less efficient amplification, which may be due to a range of factors. One possibility is lower levels of PrP^{Sc} in these samples, as suggested by negative western blot, or possibly the presence of inhibitors such as blood, which may vary between individuals. Alternatively, it could be that the reindeer CWD strain amplifies less readily in bank-vole recombinant substrate under our RT-QuIC conditions. However, bank vole substrate has shown an ability to amplify a wide range of prions [96], and overcome the strain related difference in amplification of CWD samples from both North American and Norwegian CWD isolates [95].

Whilst western blotting failed to reveal any further positive mice inoculated with Nor-R and Can-M isolates, RT-QuIC analysis did produce a borderline positive result from one of three Nor-R inoculated mice that were negative by IHC and western blotting. Whilst this inconclusive result would need to be

repeated to produce more certainty, it may be the case that further testing of brain tissue from animals negative by traditional tests would reveal more mice with subclinical CWD, as was the case in RT-QuIC analysis of Norwegian Reindeer [95].

Western blotting of mouse brains from CWD transmission experiments proved difficult despite the efforts taken to concentrate PrP^{Sc} in samples before analysis. This may have been due to the low levels of PrP^{Sc} in the samples, especially in samples with discrete localised PrP^D deposits in the brain. This was also the case in bank vole adapted CWD, where PrP^{Sc} levels in the brain were much lower than usually found in vole-adapted prion strains, for example at least 30 fold lower than the scrapie strain SS7b [135]. Electrophoretic mobility patterns of mouse-adapted CWD appeared to similar to those previously reported [253, 319] and did not differ between VM, RIII and C57BL/6 mice with Can-WT. In the Nor-M mouse with a clear western blot profile, the PrP^{Sc} appeared to have slightly lower molecular weight than scrapie controls and previously blotted Can-WT inoculated mice, which could indicate a difference in strain. Unfortunately attempts to reproduce this clear signal alongside other CWD samples for direct comparison failed, possibly due to degradation of the sample.

The diagnosis of CWD-specific PrP^D deposition in the brains of experimentally inoculated wild-type mice was complicated by the presence of unusual staining patterns and deposits that in some cases appeared very similar to PrP^D (see Figure 4-11). As the Norwegian CWD isolates are novel, all possible presentations of prion protein deposition had to be considered. Western blotting and RT-QuIC analysis of mouse brains with this staining failed to provide any evidence to support the presence of disease-associated PrP, which provides confidence that this staining was not associated with CWD transmission. Interestingly, very similar patterns of labelling have been described in reports of experimental transmission of CWD to *Cynomolgus* macaques [275]. In that study, the staining was identified with three different anti-PrP antibodies, in both inoculated and uninoculated controls, leading the

authors to draw the conclusion that it may represent areas of increased PrP^C or senPrP^{Sc} expression and/or aggregation. Increased labelling of PrP has also been reported in sheep with a variety of non-prion related neurological disorders [50].

Lymphotropism is an important feature of CWD and scrapie in natural hosts, and is linked to horizontal transmission of prion disease between animals with these diseases [450]. CWD strains appear to have variable lymphotropic behaviour; North American cervids and Norwegian reindeer display considerable PrP accumulation in lymphoid tissues, whereas the Norwegian moose cases do not [17, 102, 226, 343, 436, 450]. The lymphoid system provides a different environment for prion replication, and strain tropism in the spleen appears to be, at least in part, related to levels of PrP expression and glycosylation [153, 425, 451]. Comparative analysis of the prions in the nervous and lymphoid tissues of cervids provided evidence of multiple strains within an individual animal, with different strains harboured in the brain and spleen [450]. The lymphoid system and CNS have different tolerances for cross-species transmission, and prion strains that typically replicate poorly in the brains of transgenic mice have been shown to accumulate in the spleens. This included the transmission of Elk CWD into ovine PrP expressing mice, where the proportions of PrP^{Sc} positive mice were 9% and 94% in the brain and spleen respectively [108]. Splenic expression of PrP^C may vary in the various transgenic mice used to study CWD transmission, making comparison difficult. However, splenic PrP^D deposition has been reported in VM mice inoculated with Korean CWD [253], transgenic mice overexpressing murine PrP (tg20) [440], and transgenic mice overexpressing deer PrP (Tg1536) [442].

Using immunohistochemistry protocols to detect PrP^D deposition, varying intensities of staining were identified in the spleens of mice inoculated with Norwegian CWD isolates, which localised to the germinal centres and follicular dendritic cell networks. To confirm if this detected prion protein was PK resistant, either paraffin-embedded tissue (PET) blot, or western blotting could be performed. Optimisation of the PET blotting protocol was judged to be

difficult to the lack of spleen controls that were properly matched both in tissue fixative and formic acid treatment, which can affect the detection of PrP^D [452]. We therefore chose to analyse PK-digested, NaPTA precipitated spleen samples by western blotting, and followed this up with RT-QuIC analysis for more sensitive detection of PrP^{Sc}. NaPTA pre-treatment of spleen samples was found to improve RT-QuIC amplification of mouse spleen samples and was therefore used throughout. Norwegian mice with apparent PrP^D in the spleen were negative by western blotting, and had limited, borderline or no seeding activity in RT-QuIC. One sample (Nor-M5) that showed seeding activity at the 10⁻² dilution in two wells after a lag time of 35 hours in the first experiment, was negative upon repeat. This suggests that the borderline results here may be the result of spontaneous conversion, or possibly that the results are inconsistent, as has been previously reported for NaPTA pre-treated lymphoid tissues [300]. The deposition seen in the spleens by IHC therefore may be PrP^C which is not fully destroyed by the tissue pre-treatments. Further repeats would be necessary to more confidently determine the nature of this staining.

Unexpectedly, efficient seeding activity in RT-QuIC was consistently present in the spleens of all Can-WT inoculated mice which were also positive in the brain. This was despite negative results using IHC, suggesting that PrP^{Sc} was present, but below the level of detection for IHC and western blotting in spleens. Analysis of mice inoculated with Norwegian CWD isolates with evidence of positivity in the brains by IHC/WB/RT-QuIC failed to show any seeding activity in the spleens of these animals. Interestingly, a mouse inoculated with the Can-M isolate showed convincing evidence of seeding activity in the spleen (4/4 wells), despite being negative in the brain by IHC and western blot. This suggests that there may be a subset of mice in the Can-M transmission that have accumulated PrP^{Sc} in lymphoid tissues, although to a lower level than in previous studies of CWD transmission in overexpressing transgenic mice [108]. Testing of larger numbers of mice would be necessary to determine if this may also be true of the Norwegian transmissions, though this preliminary evidence does not suggest so. Further RT-QuIC experiments would not only allow

increased certainty of borderline results, and higher numbers of mice tested, but may also allow the discrimination of different prion strains. Amplification of prion strains using different RT-QuIC recombinant substrates can be used to differentiate between classical and atypical L-type BSE, classical and atypical scrapie in sheep, and sporadic and variant CJD in humans [96, 453–455].

Other future experiments could include repeating western blots with the SAF84 (aa 167-173) antibody, or other C-terminal antibody. SAF84 recognises a more extreme C-terminal epitope than 6H4 (aa 147-155) and is able to detect both the main C-terminal PrP^{Sc} fragment and a shorter C-terminal fragment (and its two glycosylated forms) between 10 and 14kDa not detected by other antibodies. SAF84 has been used to characterise Norwegian moose CWD, as well as Norwegian and North American CWD strains adapted in bank voles [343, 346]. It would be interesting to see if this lower fragment would be maintained upon passage to wild-type mice, as was the case in bank voles. It is possible that the use of SAF84 may also reveal IHC staining in the two positive Nor-M inoculated mice, as the PrP IHC labelling in the moose was found to be more intensive and widespread using this antibody [343]. Unfortunately due to time constraints, the weak western blot signals obtained with 6H4 antibody, and the very limited amount of available material from primary passage, these western blots were not performed. Ultimately, further mouse-to-mouse passage (secondary passage) would be required to stabilise the strains isolated upon passage to wild-type mice. This would include a shortening of the survival time, due to the adaptation of CWD to a new host species, increased attack rates and more significant neuropathology, all of which would provide more material for the analysis and discrimination of prion strains. Once adapted and stabilised, comparison to other historical transmissions in wild-type mice panels may be performed, which may provide insights into the similarities and differences of CWD and other prion diseases.

5 Final discussion

The emergence of CWD in Norway in 2016 [17] makes it vital to understand CWD in a European context; the threat posed depends on the susceptibility of European deer species, how rapidly the disease may spread, and how likely it is that cross-species transmission may occur, all of which are likely to be strongly influenced by the specific prion strains causing CWD in Europe. In this project, I therefore aimed to investigate the extent of *PRNP* genetic variability in British cervid species, compare this with other European and North American populations, and to perform *in-vitro* assays to predict the effects of amino acid polymorphisms unique to European cervid species on CWD susceptibility. In addition, I aimed to characterise and compare the strain-specific pathological phenotypes of Norwegian and Canadian CWD isolates following transmission to a panel of inbred wild-type mouse lines, as part of ongoing strain-typing studies for European CWD.

In North America, where CWD has been expanding for over 50 years, the *PRNP* genetics of cervid species and their role in CWD has been well described [290, 311] whilst knowledge of *PRNP* variation in European deer populations is limited [306, 355, 375]. To address this, we performed a large-scale survey of over 1000 British cervids including red, roe, sika, fallow, muntjac and Chinese water deer, and described the variation in red deer allele frequencies in different areas of the UK in comparison with Norway and the Czech Republic [305]. Since 2016, surveys of *PRNP* sequences in Spanish red, roe and fallow deer [336] as well as analysis of the Norwegian reindeer from the infected Nordfjella population [310] have been reported. The *PRNP* gene is extremely well conserved in cervid species and based on results presented in this thesis and previously published materials, a significant number of the UK deer population, and by extension European deer populations, would likely be susceptible to CWD strains prevalent in North America. However, polymorphisms were identified in British deer species, particularly red deer, which have unknown effects on CWD susceptibility and warrant further study. This is based on either their similarity to known polymorphisms associated with reduced CWD susceptibility in other species (for example the T98A

polymorphism in red deer which occurs close to important Q95H and G96S polymorphisms in white-tailed deer), or their position at critical sites in the prion protein structure. Polymorphisms located in the β 2- α 2 loop of the prion protein (for example P168S in red deer) are of particular interest due to the importance of this region in cross-species transmission [137, 142, 312] and the effects of similar polymorphisms in sheep (P168L) and reindeer (V169M and N176D) on prion disease susceptibility and conversion *in-vitro* [75, 241, 242, 301, 310, 373]. It cannot be ruled out that additional *PRNP* polymorphisms are present at low frequency in UK deer populations, not detected by our study. This is more likely for species where fewer individuals could be sourced for analysis, such as Chinese water deer.

The effects of *PRNP* polymorphisms in European cervid species on CWD susceptibility remain unresolved due to the lack of natural cases in these species; the disease is currently limited to small numbers of reindeer, moose and red deer in Scandinavian countries. *In-vitro* PrP conversion assays are a useful alternative that have broadly been shown to recapitulate the effects of *PRNP* variation and species barriers demonstrated by experimental challenge or natural infection [74, 75, 279, 301]. To investigate the effects of polymorphisms found in British red deer (T98A, P168S) and fallow deer (138N) populations, the aim was to produce cell lines expressing these PrP variants to use as substrate for the *in-vitro* conversion assay, PMCA. Initially, red deer TPQ PrP tagged with the 3F4 epitope was expressed in RK13 cells to allow specific detection of PrP converted in PMCA using the 3F4 antibody. Unfortunately, the PrP^C expression levels in cervid PrP-expressing cell lysates were not sufficiently high to allow efficient conversion in PMCA reactions, in accordance the experience of another group, who showed that bulk culture of ovine PrP-expressing cells and/or addition of PrP-null mouse brain homogenates were necessary to achieve scrapie amplification in cell lysate substrate [79]. Further development on this cell line to increase expression levels may be useful for future experiments comparing conversion of different cervid *PRNP* sequences *in-vitro*. Alternative approaches include the use of gene-edited cell lines [408],

or production of recombinant protein for comparative analysis in RT-QuIC [301] or in-silico analysis [304, 456]. However, the simplicity of these assays compared to *in-vivo* infections should always be considered, as they do not take factors such as route of exposure, dose, and host-specific cofactors into account. Prediction of the effects of cervid *PRNP* variants on CWD susceptibility using a variety of *in-vitro* and *in-silico* approaches is currently being actively pursued by a number of research groups in North America and Europe. These assays may be useful for preliminary screening of *PRNP* variants that impact CWD susceptibility, which could then be further analysed in transgenic mice or the natural host. It is hoped that identification of polymorphisms associated with reduced susceptibility will inform modelling of CWD transmission in cervid populations, as well as have potential for selective breeding management. However, for the latter several obstacles are present, including the feasibility of this approach in wild animals, and the association of some *PRNP* variants with the potential for increased periods of preclinical infectious shedding into the environment [226]. Additionally, *PRNP* variants are becoming increasingly recognised in the emergence of new CWD strains [155, 302, 318, 323].

In-vitro analyses are often accompanied by *in-vivo* experiments in small rodent models. Experiments in transgenic mice expressing a variety of North American cervid *PRNP* sequences have provided evidence of the effects of polymorphisms on CWD susceptibility, as well as evidence for distinct CWD strains [149, 302, 304, 318, 320, 323, 328]. *PRNP* polymorphisms may affect susceptibility to prion diseases in a strain-specific manner and drive the adaptive diversification and emergence of novel prion strains [155, 304, 328]. In addition to understanding the *PRNP* variation in European cervids, it is therefore important to understand the strains present in European CWD cases. A number of strain-typing experiments undertaken via bioassay in small rodent models are still ongoing, and only transmissions to bank voles, the most rapid bioassay model for CWD [135], have so far produced published evidence of different strains in Norwegian compared to North American CWD isolates [346]. Transmission of Norwegian reindeer isolates was inefficient, however serial passage produced a

bank vole adapted strain with slower disease kinetics and different PrP^{Sc} conformation to other strains. This, in addition to the failure to re-isolate the 'fast' CWD isolated from all passaged North American isolates to date [135], has been used to argue for the presence of a different CWD strain in Norwegian reindeer [346]. It is unclear whether the reported *PRNP* genetic variability in reindeer may have an impact upon CWD strains within the Nordfjella population. The 225Y allele in reindeer was under-represented in cases (13.2%) compared to controls (33.7%)[310], and considering the reduced susceptibility of mule deer expressing 225F and differences in clinical disease in 225F heterozygous deer infected with CWD, it may be interesting to further characterise these cases [233]. The cases identified in Scandinavian moose are unusual and, based on neuropathological and biochemical characteristics, are unlike any CWD isolate previously described [343]. Analysis of three moose isolates from Norway produced evidence of at least two different strains, with phenotypes distinct from those found in Norwegian reindeer and North American isolates. Moose CWD adapted in bank voles shared neuropathological and biochemical features consistent with what was observed in the natural host, which suggests that these atypical disease features are due to the manifestation of prion strain properties [343, 346].

Strain typing in conventional wild-type mouse models allows comparison of results to historical datasets for a wide range of different prion isolates [146], and serial passage of North American CWD isolates in wild-type mice has produced the mouse CWD strain 409V (Diack, pers. comm. [424]). In this project, primary passage of Norwegian (moose and reindeer) and Canadian CWD isolates in wild-type mice produced low transmission rates, consistent with a strong species barrier, for both Norwegian isolates and one of two Canadian isolates. However, the second Canadian isolate, an experimental CWD isolate from white-tailed deer expressing the 95H/96S genotype, transmitted relatively efficiently in three lines of wild-type mice (VM, C57, RIII). This is in agreement with previously published results where transmission was successful in 7/7 C57BL/6 mice [319]. This experimental isolate from a

95H/96S genotype deer has been reported to contain a mixture of Wisc-1 and H95+ strains, which are differentially isolated in tg33 (wild-type deer) and tg60 (96S) transgenic mice [318]. Isolates containing only the Wisc-1 strain failed to transmit to wild-type C57BL/6 mice [319], suggesting that the increased transmission efficiency in wild type mice observed was a feature of the H95+ strain. The biological characterisation of Norwegian and Canadian isolates described in this thesis was limited by low levels of transmission for three of four isolates, which limited the materials available for analysis. However higher attack rates on continued sub-passage in the same mouse lines would be expected to provide more reliable and consistent data for strain characterisation.

Determining the threat CWD poses in Europe hinges on a considerable number of uncertainties. If the Norwegian reindeer cases, (though distinct from North American CWD based upon strain typing) are contagious in nature as suggested by the epidemiology and the peripheral distribution of PrP^{Sc} in lymphoid tissues [17], horizontal transmission and environmental contamination of prions can be assumed to occur. Subsequent spread of infection would therefore be likely, and the risk-factors for this spread have been outlined in a recent EFSA report [344]. The nature of the cases in moose and red deer are uncertain, however they have been widely described as probably non-contagious and representative of spontaneous disease in these species [343, 350]. This is based on the advanced age (>10 years) of affected animals, and the apparent lack of lymphoid involvement, which is also seen in atypical forms of scrapie and BSE [19]. If this is correct, the potential for horizontal transmission and environmental contamination would be minimal in live animals, however it is important to consider that degrading carcasses could still act as a source of environmental contamination. The identification of strain variation between individual moose is consistent with the observation of multiple strains of atypical BSE [16, 346] and fits with the theory of a spontaneous aetiology in these animals. Support for spontaneous or 'sporadic' CWD has been provided by the *de-novo* generation of a novel prion strain from cervid PrP subjected to PMCA amplification [173]. This

raises the question of whether increased surveillance in aged cervids could identify further cases of CWD in other cervid species and geographical locations. Surveillance in North America has not yet identified such cases, however it is often limited in analysis and surveillance often focuses on the detection of PrP^{Sc} in lymphoid tissues and may miss cases where only the CNS is affected [228]. Whether spontaneous cases could transmit to susceptible species, perhaps even gaining more classical contagious phenotypes, remains speculative. However, both atypical scrapie and H-BSE have given rise to phenotypes typical of classical BSE, following intracerebral inoculation of transgenic mice expressing bovine PrP and wild-type mice respectively [172, 457].

Many uncertainties remain regarding CWD strains. Whilst bioassay in small rodent models is currently the gold-standard for discrimination of prion strains, these studies are not without drawbacks. The use of overexpressing transgenic mice has allowed transmission of a variety of prion sources; however, artefactual effects of transgene overexpression and strain mutation complicate the interpretation of results. Gene-targeted mice address some of these issues and also allow analysis of the peripheral pathogenesis of CWD [320]. The number of CWD strains circulating in North America, whether subdivided based on cervid species or geographical location, has not yet been determined. An assessment of different CWD isolates from a range of species and geographical locations performed by Angers and colleagues [149] provided evidence of two different CWD strains (CWD1 and CWD2), however further experiments have identified further distinct strains in white-tailed deer expressing 95H [318] and elk expressing 132L [323]. Recent work has employed a range of conformational assays and phenotypic comparisons in transgenic mice to show that transmission of CWD prions in hosts with novel PrP^C sequences drives the adaptive diversification of prion conformations, which can lead to the competitive selection and emergence of new prion strains [155]. The selective pressure upon prions when in a novel host environment is largely a product of the host *PRNP* genotype. The new prion conformers formed may be non-adaptive and unstable, accumulating to low levels (as was the case for S96 PrP)

or emerge as a novel prion strain (H95+ strain emerged in hosts expressing 95H-PrP^C). This work underscores the importance of understanding *PRNP* variation in cervid species not just for the prediction of susceptibility, but also for the potential emergence of new prion strains. For example, it is possible that the increased frequency of the Leu-132 haplotype in elk in CWD endemic areas may have led to the emergence of a new prion strain (as supported by [323]), which may explain the lack of association of the 132L polymorphism with reduced susceptibility in one study [289]. While speculative, this highlights that the protective effects of *PRNP* polymorphisms in cervids are not always fixed and are dependent upon the specific strains of CWD circulating.

Understanding strain variation is key as the strain properties of the infectious agents, as well as the differences in the PrP primary structure between the host and donor species, impact the transmissibility of prion diseases across species barriers. Therefore, different CWD strains may have different potential for cross-species transmission to other livestock species and humans. The cross-species transmission potential of North American CWD has been well studied (reviewed in [458]), in particular the potential for CWD contaminated environments to give rise to novel prion diseases in other livestock species. Low rates of transmission have been reported for cattle and sheep intracerebrally inoculated with CWD [263–267], results in transgenic mice overexpressing ovine or bovine PrP produced negative or low levels of PrP^{Sc} in the CNS [108, 268], but much higher levels of asymptomatic replication in spleen tissue of transgenic mice expressing ovine VRQ PrP challenged with CWD have been reported [108]. Interestingly despite a significant species barrier, similar low levels amplification of CWD in the CNS and lymphoid tissues of pigs has been reported following oral CWD challenge, raising the possibility of a reservoir of infection in naturally exposed pigs [252]. Experiments to assess the cross-species transmission potential of Norwegian CWD isolates are ongoing.

The zoonotic potential of CWD is a contentious and widely discussed topic. A number of studies investigating the ability of the CWD agent to cross the human species barrier have been carried out through experimental inoculation of non-

human primates, transgenic mice expressing human prion protein, and *in-vitro* techniques (reviewed in [16, 279, 458]. Experimental transmission of CWD to squirrel monkeys has been achieved, whilst challenge of *Cynomolgus* macaques (evolutionarily closer to humans) has provided conflicting results [249, 275, 278]. The reasons for the differences between these two *Cynomolgus* macaque studies remain to be resolved, however could be due to differences in the CWD strains present in the inocula used, or the dosing/infection protocol used. Interestingly, the potential for CWD strain to impact cross-species transmission has been previously demonstrated [319], and is supported by the efficient transmission of the H95+ strain we observed in wild-type mice. Species such as mice and humans with a flexible $\beta 2$ - $\alpha 2$ loop were previously hypothesised to be resistant to rigid loop CWD prions, [136, 142], however these findings raise concern for emerging CWD strains with increased zoonotic potential [319].

In-vitro analyses support the capacity for the human prion protein to be misfolded by CWD prions, with conversion demonstrated in cell lines, PMCA and RT-QuIC assays [279, 280, 282, 283]. A recent study by Barria and colleagues highlighted the efficient PMCA conversion of human prion protein seeded with reindeer CWD of the same *PRNP* genotype as cases reported in Norway [282, 310]. Counter to this, a number of experiments carried out in transgenic mice expressing human prion protein (129MM, MV or VV), have failed to produce convincing evidence of transmission with North American isolates (reviewed in [344]). A recent report has likewise described the resistance of humanised transgenic mice to Norwegian reindeer and moose isolates [439]. However, these studies did not carry out blind secondary passages, which have previously been necessary to demonstrate the transmissibility of scrapie and C-BSE isolates in similar models [459]. It should be noted that often these experiments do not involve examination of the spleen, which was also the case in the recent Norwegian transmission [439] due to low levels of PrP expression in spleens of some transgenic mouse lines. Importantly, subclinical transmission of CWD has been reported in humanised mice with splenic involvement [344], which is similar to previous studies examining the spleens of humanised mice with BSE

[108]. Interestingly, we observed RT-QuIC seeding activity in the spleens of mice also showing positivity in the CNS and clinical signs following transmission with the Canadian H95+ strain isolate, which indicates lymphoid involvement may have been important for cross-species transmission of CWD. In light of the limited epidemiological data and prolonged incubation times in human disease, the inconsistent findings from *in-vitro* and *in-vivo* studies and the impact of prion strain variation on cross-species transmission potential, it seems prudent to consider the zoonotic transmission of CWD a risk until proven otherwise.

Future studies investigating CWD in Europe will build upon the work presented in this thesis and include genetic surveys of *PRNP* variation in other European cervid populations. Following from this, the effects specific prion protein variants will then be assessed using *in-vitro* models similar to those presented, employing either cervid-PrP^C expressing cell lines or the use of recombinant protein production systems. This will allow for the estimation of CWD susceptibility in European cervid populations, which in turn will inform modelling of CWD spread, risk assessments and management plans.

Additionally, it will be crucial to understand the effects of genetic variation in cervid populations considering the mutable and evolving nature of prion strains, which may arise in new host genetic backgrounds. Ongoing and additional strain typing studies will help to reveal the differences between European (Norwegian) and North American CWD strains, as well as the potential for these prions to cross species barriers. Increased surveillance as well as targeting of species other than reindeer and moose, such as red deer, will improve our understanding of the true prevalence/incidence of CWD in Europe. Targeted surveillance in aged cervids may be interesting to determine if 'unusual' or 'atypical' CWD occurs spontaneously in any cervid population or geographic location.

In conclusion, the work presented in this thesis represents some of the first steps in a longer journey towards understanding the prion protein gene variability in European cervid species, predicting the effects of these variants on CWD susceptibility and the diversification of CWD strains in Europe.

6 References

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7 Appendix

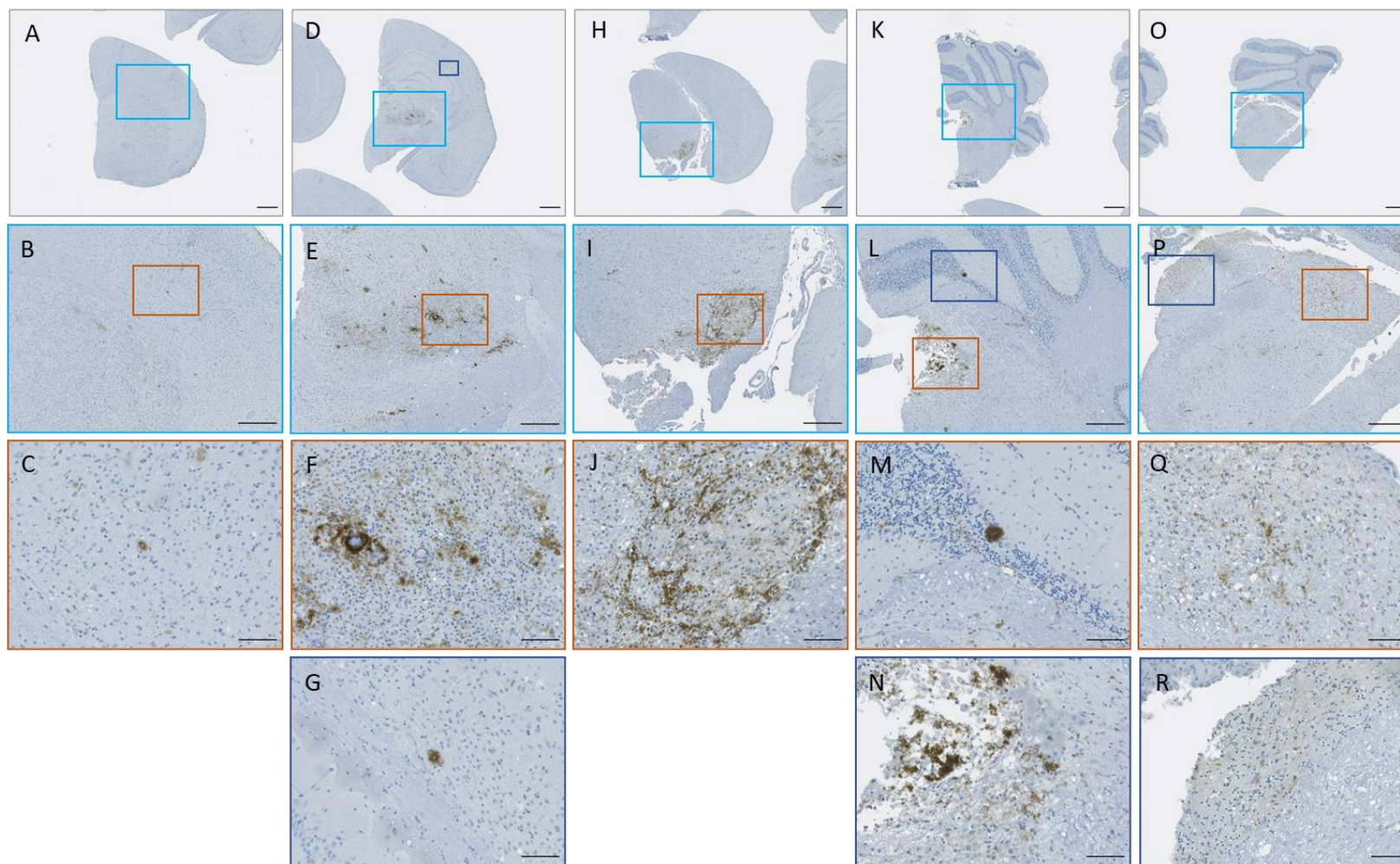


Figure 7-1: Representative PrP^D deposition in the brain of a VM mouse inoculated with Can-WT isolate.

Brain regions depicted: C,G, cortex; F, thalamus; J, substantia nigra; M, cerebellum; N, locus coeruleus; Q, medulla; R, cochlear nucleus.
Scale bars: A,D, H, K, O, 500 μ m. B,E,I,L,P 200 μ m. C, F, J, M,Q,G,N,R, 50 μ m

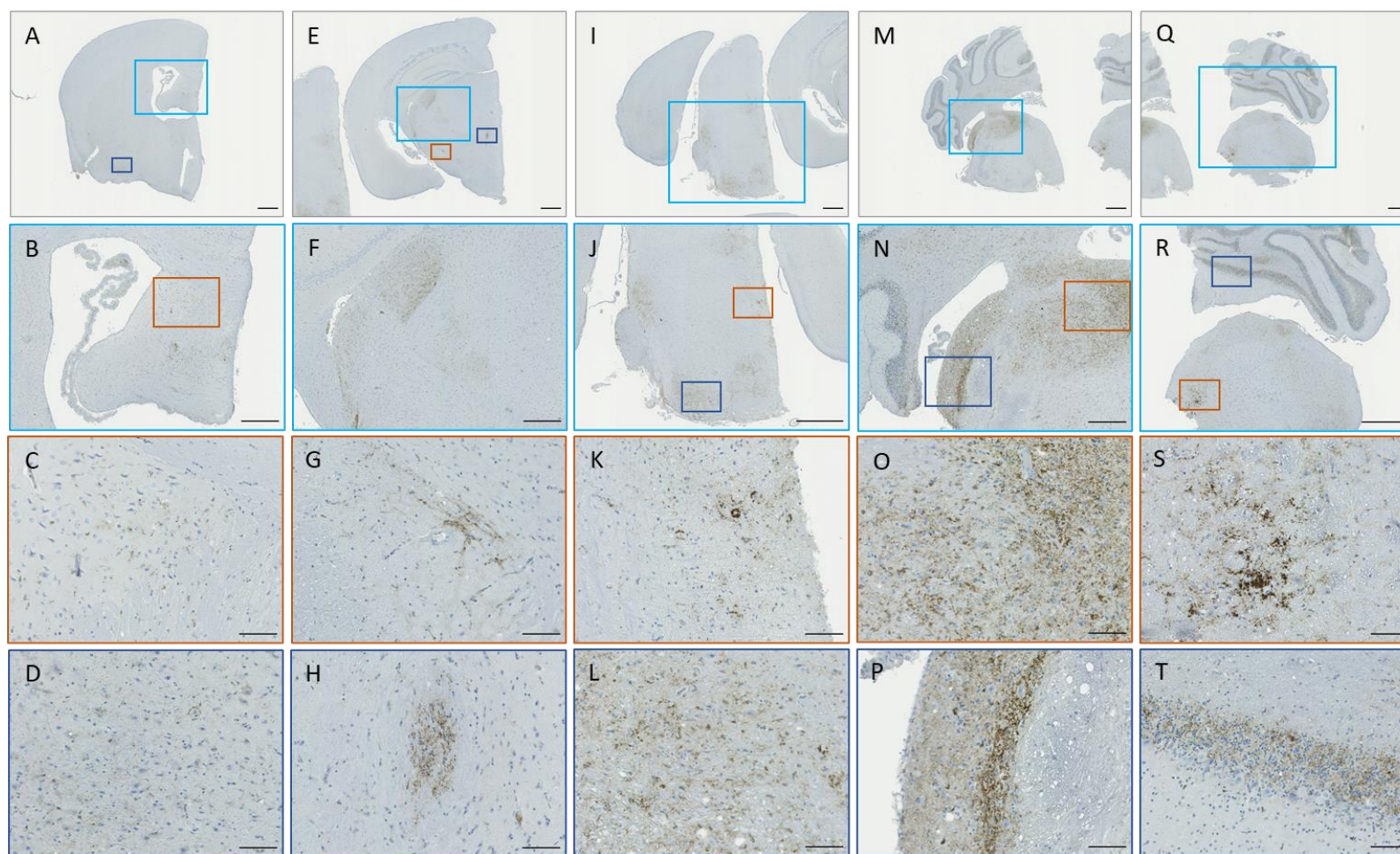


Figure 7-2: Representative PrP^D deposition in the brain of a C57BL/6 mouse inoculated with Can-WT isolate.

Brain regions depicted: C,D, striatum; F, dorsal lateral geniculate nucleus, G, substantia nigra; H, fasciculus retroflexus, K, dorsal raphe; L, ventral pons; O, vestibular nuclei, P, cochlear nucleus, S, medulla; T, cerebellum.

Scale bars: A,E,I,M,Q,B,F,N 500µm. I,Q 200µm. C,G,K,O,S,D,H,L,P,T 50µm

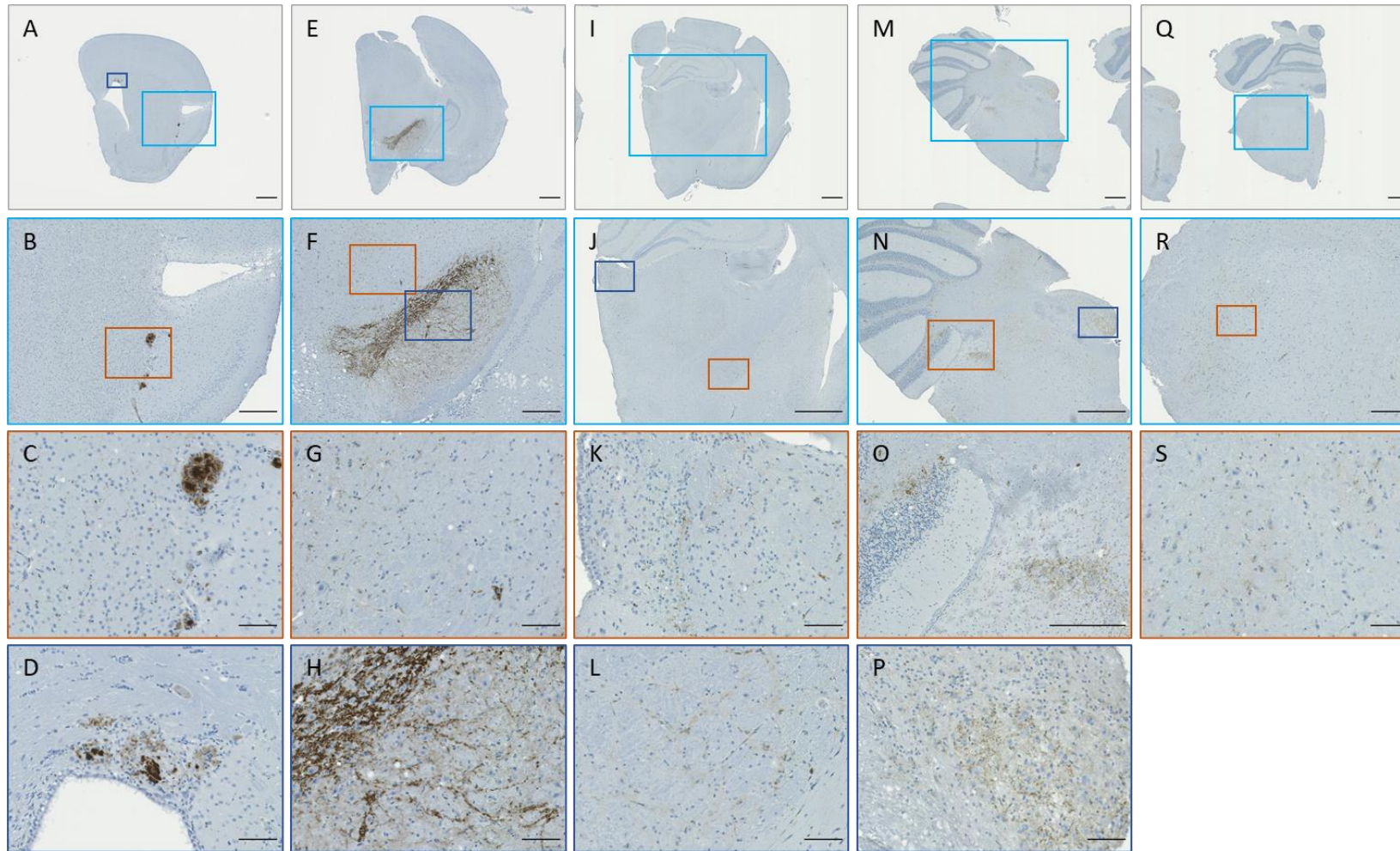


Figure 7-3: Representative PrP^D deposition in the brain of an RIII mouse inoculated with Can-WT isolate.

Brain regions depicted: C, cortex, D, lateral ventricle/corpus callosum, F, substantia nigra; J, thalamus; K, habenular nucleus; O, cerebellum/locus coeruleus, P, cochlear nuclei, R, medulla.

Scale bars: A,E,I,M,Q, J, N: 500μm, B,F,R: 200μm, C,G,K,O,S,D,H,L,P: 50μm.

Table 7-1: Overview of transmission results for Norwegian CWD isolates in wild-type mice

Mouse details				Transmission results								
Isolate	Mouse line	Mouse number	Sex	Survival ¹	Clinical signs	Vac (Br)	IHC (Br) ²	WB (Br)	Rt-QuIC (Br)	IHC (Sp) ³	WB (SpN+)	Rt-QuIC (SpN+)
Nor-R	VM	M00109293	M	600	N	N	N	N	-	0	-	-
	VM	M00109294	M	600	N	N	N	N	-	0	-	-
	VM	M00109295	M	600	N	N	N	N	-	0	-	-
	VM	M00109296	M	574	N	P	N	N	N	0	-	-
	VM	M00109297	M	574	N	N	N	N	-	0	-	-
	VM	M00109299	F	596	N	N	N	N	-	0	-	-
	VM	M00109300	F	596	N	N	N	N	-	0	-	-
	VM	M00109504	F	596	N	N	N	N	-	>10/++ +	N	B
	VM	M00109505	M	582	N	N	N	N	-	0	-	-
	VM	M00109506	M	589	N	N	N	N	-	0	-	-
	VM	M00109507	M	582	N	N	N	N	-	0	-	-
	VM	M00109508	M	600	N	N	N	N	-	0	-	-
	VM	M00109652	F	596	N	N	N	N	-	0	-	-
	VM	M00109653	F	596	N	N	N	N	-	1/+	-	-
	VM	M00109772	F	600	N	N	N	N	-	1/+	-	-
	VM	M00109773	F	600	N	N	N	N	-	0	-	-
	VM	M00109774	F	600	N	N	N	N	-	2/+++	-	-
	VM	M00109775	F	600	N	N	N	N	-	0	-	-
Nor-R	C57BL/6	M00111886	M	596	N	N	N	N	-	1/++	-	-
	C57BL/6	M00111887	M	596	N	N	N	N	-	0	-	-
	C57BL/6	M00111888	M	596	N	N	N	N	-	0	-	-
	C57BL/6	M00111889	M	596	N	N	P/+	N	P	0	-	N
	C57BL/6	M00111890	M	596	N	N	N	N	-	0	-	-

	C57BL/6	M00111905	M	596	N	N	na	N	-	na	-	-
	C57BL/6	M00111906	M	412	N	N	N	N	-	1/+	-	-
	C57BL/6	M00111907	M	596	N	N	N	N	N	>10/++	N	N
										+		
	C57BL/6	M00111908	M	552	N	N	N	N	-	3/+	-	-
	C57BL/6	M00111909	F	600	N	N	N	N	-	6/+++	-	-
	C57BL/6	M00111910	F	574	N	P	N	N	-	2/+	-	-
	C57BL/6	M00111911	F	600	N	N	N	N	-	1/+	-	-
	C57BL/6	M00111912	F	600	N	N	N	N	-	4/+	-	-
	C57BL/6	M00111913	F	600	N	N	N	N	-	2/+	-	-
	C57BL/6	M00111923	F	601	N	N	N	N	-	0	-	-
	C57BL/6	M00111925	F	601	N	N	P/+	N	P	0	-	N
	C57BL/6	M00111926	F	601	N	N	N	N	-	0	-	-
Nor-R	RIII	M00106794	M	606	N	N	N	N	-	2/+	-	-
	RIII	M00106795	M	606	N	N	N	N	-	1/+	-	-
	RIII	M00106967	M	282	N	N	N	N	-	na	-	-
	RIII	M00106969	M	262	N	N	N	N	N	0	-	-
	RIII	M00112019	M	600	N	N	N	N	-	0	-	-
	RIII	M00112020	M	600	N	N	N	N	-	0	-	-
	RIII	M00112021	M	119	N	N	N	N	-	0	-	-
	RIII	M00112022	M	600	N	N	N	N	-	0	-	-
	RIII	M00112024	F	526	N	N	N	N	-	0	-	-
	RIII	M00112025	F	287	N	N	N	N	-	0	-	-
	RIII	M00112026	F	582	N	N	N	N	-	4/+	-	-
	RIII	M00112272	F	600	N	N	N	N	-	2/+	-	-
	RIII	M00112273	F	540	N	N	N	N	-	0	-	-
	RIII	M00112274	F	600	N	N	N	N	-	>10/+	-	N
	RIII	M00112275	F	600	N	N	N	N	-	0	-	-

Nor-M	VM	M00110620	M	600	N	N	N	N	-	0	-	-
	VM	M00110621	M	561	N	N	N	N	-	0	-	-
	VM	M00110622	M	600	N	N	N	N	-	0	-	-
	VM	M00110623	F	600	N	N	N	N	-	0	-	-
	VM	M00110625	F	517	N	N	N	N	-	0	-	-
	VM	M00110626	F	600	N	N	N	N	-	0	-	-
	VM	M00110628	M	600	N	N	N	N	-	1/+	-	N
	VM	M00110629	F	600	N	N	N	N	-	0	-	-
	VM	M00110749	F	600	N	N	N	N	-	0	-	-
	VM	M00110750	F	600	N	N	N	N	-	0	-	-
	VM	M00110751	F	590	N	N	N	N	N	0	-	-
	VM	M00110752	F	600	N	N	N	N	-	0	-	-
	VM	M00110826	M	573	N	N	N	N	-	0	-	-
	VM	M00110827	M	597	N	N	N	N	-	0	-	-
	VM	M00110828	M	597	N	N	N	N	-	0	-	-
	VM	M00110830	F	597	N	N	N	N	-	0	-	-
Nor-M	C57BL/6	M00111891	M	597	N	N	N	N	-	0	-	-
	C57BL/6	M00111892	M	597	N	N	N	N	-	0	-	-
	C57BL/6	M00111893	M	597	N	N	N	N	-	0	-	-
	C57BL/6	M00111894	M	597	N	N	N	N	-	0	-	-
	C57BL/6	M00111895	M	597	N	N	N	N	-	0	-	-
	C57BL/6	M00111901	M	601	N	N	N	N	-	>10/+++	N	B
	C57BL/6	M00111902	M	469	N	N	N	N	-	0	-	-
	C57BL/6	M00111903	M	601	N	N	N	N	-	7/++	-	N
	C57BL/6	M00111904	M	601	N	N	N	P	P	0	-	-
	C57BL/6	M00111914	F	600	N	N	N	N	-	0	-	-
	C57BL/6	M00111915	F	568	N	N	N	N	-	0	-	-

	C57BL/6	M00111916	F	600	N	N	N	N	-	0	-	-
	C57BL/6	M00111917	F	600	N	N	N	N	-	0	-	-
	C57BL/6	M00111918	F	600	N	N	N	N	-	0	-	-
	C57BL/6	M00111919	F	600	N	N	N	N	-	0	-	-
	C57BL/6	M00111920	F	600	N	N	N	N	-	0	-	-
	C57BL/6	M00111921	F	600	N	N	N	N	-	3/+	-	-
	C57BL/6	M00111922	F	600	N	N	N	N	-	0	-	-
Nor-M	RIII	M00108891	M	138	N	N	N*	na	na	na	na	na
	RIII	M00108892	M	394	N	N	N	N	-	0	-	-
	RIII	M00108893	M	599	N	N	N	N	-	0	-	-
	RIII	M00111068	F	575	P	P	N	P	P	0	-	N
	RIII	M00112012	M	342	N	N	N	N	-	0	-	-
	RIII	M00112013	M	600	N	N	N	na	na	0	-	-
	RIII	M00112014	M	600	N	N	N	N	N	0	-	-
	RIII	M00112015	M	465	N	N	N	N	-	0	-	-
	RIII	M00112327	F	475	N	N	N	N	-	2/+	-	-
	RIII	M00112328	F	181	N	N	N	na	na	na	na	na
	RIII	M00112503	M	577	N	N	N	na	na	na	na	na
	RIII	M00112504	M	363	N	N	N	N	-	0	-	-
	RIII	M00112505	F	600	N	N	N	N	-	>10/+	-	-
	RIII	M00112507	F	600	N	N	N	N	-	0	-	N

Abbreviations: P=Positive, N=Negative, '-' = not tested, na = tissue not available. Br= Brain tissue, Sp=Spleen tissue SpN+= NaPTA precipitated spleen material. *=Haemosiderin

¹ Survival is expressed as days post-inoculation ²IHC (Br) results are given as negative or positive/semiquantitative score (+ to +++) ³IHC (Sp) results are given as number of follicles with staining/intensity of staining (+ to +++)).

Table 7-2: Overview of transmission results for Canadian CWD isolates in wild-type mice

Isolate	Mouse details			Transmission results								
	Mouse line	Mouse number	Sex	Survival	Clinical signs	Vac (Br)	IHC (Br)	WB (Br)	Rt-QuIC (Br)	IHC (Sp)	WB (SpN+)	Rt-QuIC (SpN+)
Can-WT	VM	M00130312	Female	596	N	P	P/++	-	-	-	-	-
	VM	M00130315	Female	535	P	P	P/+++	P	-	0	-	P
	VM	M00130316	Female	596	N	P	P/+	-	-	-	-	-
	VM	M00130861	Male	534	P	P	P/+++	P	-	-	-	-
	VM	M00130862	Male	595	P	N	N	-	-	0	-	-
	VM	M00130863	Male	576	P	P	P/+++	P	-	0	-	P
	VM	M00130872	Female	105	N	N	N	-	-	-	-	-
	VM	M00130873	Female	517	P	P	P/+++	P	P	0	-	-
	VM	M00130874	Female	595	P	N	P/+	-	-	-	-	-
	VM	M00130875	Female	154	N	N	N	-	-	-	-	-
	VM	M00130876	Female	553	P	N	N	-	-	0	-	-
Can-WT	C57BL/6	M00132814	Male	658	N	P	P/+++	-	-	-	-	-
	C57BL/6	M00132815	Male	519	P	P	P/++	-	-	-	-	-
	C57BL/6	M00132816	Male	616	P	P	P/+++	-	-	-	-	-
	C57BL/6	M00132817	Male	616	P	P	P/+++	P	-	-	-	-
	C57BL/6	M00132818	Male	616	P	P	P/+++	P	P	0	N	P
	C57BL/6	M00132824	Male	667	P	P	P/+++	-	-	-	-	-
	C57BL/6	M00132825	Male	609	N	P	P/+++	-	-	-	-	-
	C57BL/6	M00132826	Male	667	P	P	P/+++	P	-	0	-	P
	C57BL/6	M00132827	Male	667	P	P	P/++	-	-	-	-	-
	C57BL/6	M00132832	Female	591	N	P	P/+++	-	-	-	-	-
	C57BL/6	M00132833	Female	674	N	P	P/++	-	-	0	-	-
	C57BL/6	M00132834	Female	674	N	P	P/+	-	-	-	-	-
	C57BL/6	M00132835	Female	674	N	P	P/++	-	-	-	-	-

	C57BL/6	M00132836	Female	674	N	N	P/+	-	-	0	-	-
	C57BL/6	M00132842	Female	674	N	N	P/+	-	P	-	-	-
	C57BL/6	M00132843	Female	582	N	N	P/+	P	-	0	-	-
	C57BL/6	M00132845	Female	674	N	N	N	-	-	-	-	-
Can-WT	RIII	M00131035	Female	364	N	N	N	-	-	-	-	-
	RIII	M00131448	Male	309	N	N	N	-	-	-	-	-
	RIII	M00131449	Male	472	N	N	N	-	-	-	-	-
	RIII	M00131450	Male	658	N	N	P/+	-	-	-	-	-
	RIII	M00131451	Female	486	N	N	N	-	-	-	-	-
	RIII	M00131452	Female	658	N	N	P/+	-	-	-	-	-
	RIII	M00131453	Female	658	N	N	P/++	P	-	2/+	-	P
	RIII	M00131454	Female	540	P	P	P/++	P	-	-	-	-
	RIII	M00131457	Female	477	P	N	N	-	-	-	-	-
	RIII	M00131459	Female	304	N	N	P/+	-	-	-	-	-
	RIII	M00131464	Male	393	N	N	P/+	-	-	-	-	-
	RIII	M00131465	Male	540	P	P	P/+++	P	-	2/+	-	-
	RIII	M00131466	Male	486	N	N	N	-	-	-	-	-
	RIII	M00131467	Male	658	N	N	P/++	P	-	0	-	P
	RIII	M00131469	Male	310	N	N	N	-	-	0	-	-
Can-M	VM	M00130307	Male	609	N	N	N	N	-	0	-	P
	VM	M00130308	Male	533	P	N	N	N	-	-	-	-
	VM	M00130524	Male	574	P	N	N	na	na	na	na	na
	VM	M00130525	Male	621	P	N	N	N	-	0	-	N
	VM	M00130526	Male	621	P	N	N	N	-	-	-	-
	VM	M00130527	Male	531	N	N	N	N	-	0	-	-
	VM	M00130528	Male	247	N	N	N	na	na	na	na	na
	VM	M00130857	Female	518	N	N	N	N	-	-	-	-
	VM	M00130858	Female	587	N	N	N	N	-	-	-	-

	VM	M00130859	Female	658	P	N	N	N	-	-	-	-
	VM	M00130860	Female	670	N	P	N	N	-	-	-	-
	VM	M00130865	Female	335	N	N	N	N	-	-	-	-
	VM	M00130866	Female	308	N	N	N	N	-	-	-	-
	VM	M00130867	Female	670	N	N	N	N	-	0	-	-
	VM	M00130868	Female	670	N	N	N	N	-	-	-	-
	VM	M00131275	Female	658	P	N	N	N	-	-	-	-
Can-M	C57BL/6	M00132819	Male	659	N	N	N	N	-	0	-	N
	C57BL/6	M00132820	Male	666	N	N	N	na	na	na	na	na
	C57BL/6	M00132821	Male	666	N	N	N	N	-	-	-	-
	C57BL/6	M00132822	Male	506	N	N	N	N	-	-	-	-
	C57BL/6	M00132823	Male	666	N	N	N	N	-	-	-	-
	C57BL/6	M00132828	Male	667	N	N	N	N	-	0	-	N
	C57BL/6	M00132829	Male	667	N	N	N	N	-	-	-	-
	C57BL/6	M00132830	Male	572	N	N	N*	na	na	na	na	na
	C57BL/6	M00132831	Male	667	N	N	N	N	-	0	-	-
	C57BL/6	M00132837	Female	519	N	N	N	N	-	-	-	-
	C57BL/6	M00132838	Female	673	N	N	N*	na	na	na	na	na
	C57BL/6	M00132839	Female	674	N	N	N	N	-	-	-	-
	C57BL/6	M00132840	Female	674	N	N	N	N	-	-	-	-
	C57BL/6	M00132841	Female	674	N	N	N	N	-	-	-	-
	C57BL/6	M00132846	Female	540	N	N	N	N	-	-	-	-
	C57BL/6	M00132847	Female	674	N	N	N	N	-	-	-	-
	C57BL/6	M00132848	Female	674	N	N	N	N	-	0	-	-
	C57BL/6	M00132849	Female	674	N	N	N	N	-	0	-	-
Can-M	RIII	M00130299	Male	679	N	N	N	N	-	-	-	-
	RIII	M00130300	Male	679	N	N	N	N	-	0	-	N
	RIII	M00130302	Male	679	N	N	N	N	-	0	-	-

RIII	M00132345	Male	268	N	N	N	N	-	-	-	-
RIII	M00132346	Male	630	N	N	N	N	-	0	-	-
RIII	M00132347	Male	630	N	N	N	N	-	-	-	-
RIII	M00132348	Female	630	N	N	N	N	-	-	-	-
RIII	M00132349	Female	325	N	N	N	na	na	na	na	na
RIII	M00132350	Female	630	N	N	N	N	-	-	-	-
RIII	M00132351	Female	528	P	N	N	N	-	-	-	-
RIII	M00132480	Female	239	N	N	N	na	na	na	na	na
RIII	M00132481	Female	630	N	N	N	N	-	0	-	N
RIII	M00132482	Female	630	N	N	N	N	-	-	-	-
RIII	M00132483	Female	630	N	N	N	N	-	0	-	-
RIII	M00132484	Female	630	N	N	N	N	-	-	-	-

Abbreviations: P=Positive, N=Negative, '-' = not tested, na = tissue not available. Br= Brain tissue, Sp=Spleen tissue SpN+= NaPTA precipitated spleen material. *=Haemosiderin

¹ Survival is expressed as days post-inoculation ²IHC (Br) results are given as negative or positive/semiquantitative score (+ to +++) ³IHC (Sp) results are given as number of follicles with staining/intensity of staining (+ to +++)).