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Characterizing patterns of tissue tropism and environmental reservoirs of PrP^{CWD} in infected cervids

by

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ABSTRACT

Chronic wasting disease (CWD) is a fatal prion disease affecting cervids. Believed to be transmitted horizontally, the factors affecting CWD contagion are unknown, but bodily fluids or excretions have been implicated as possible routes of transmission. Protein misfolding cyclic amplification (PMCA) was used as an ultra-sensitive method of detection of prion protein (PrP^{CWD}) across four different cervids: white-tailed deer (WTD), elk, mule deer, and red deer. Strong correlations were observed between traditional diagnostic tests and PMCA for detection of CWD, validating the PMCA PrP^{CWD} assay as a potential diagnostic tool. Assessment of PrP^{CWD} tissue tropism and shedding of CWD prions in the secreta/excreta of infected WTD revealed high levels of PrP^{CWD} in organs other than skeletal muscle throughout the course of infection. PrP^{CWD} was detected in urine, saliva and fecal samples at high levels during the course of infection suggesting that concentrated foci of infectious prions may be sporadically present in the environment.

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LIST OF ABBREVIATIONS

BSE	bovine spongiform encephalopathy
CB	conversion buffer
CCAC	Canadian Council of Animal Care
CFIA	Canadian Food Inspection Agency
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CO_2	carbon dioxide
CWD	chronic wasting disease
Da	dalton
dH_2O	distilled water
DNA	deoxyribonucleic acid
dpi	days post inoculation
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
fCJD	familial Creutzfeldt-Jakob disease
FFI	fatal familial insomnia
fg	femtogram
Fig	figure
FSE	feline spongiform encephalopathy
g	gram
GALT	gut-associated lymphoid tissue
GAM	goat anti-mouse
GPI	glycosylphosphatidyl inositol
GSS	Gerstmann-Sträussler-Scheinker disease
hr	hour
HRP	horse radish peroxidase
IBH	infectious brain homogenate
i.c.	intracranial
iCJD	iatrogenic Creutzfeldt-Jakob disease
ID	infectious dose
IHC	immunohistochemistry
Inc.	incorporated
i.p.	intraperitoneal
kD	kiloDalton

L	litre
LRS	lymphoreticular system
mAb	monoclonal antibody
MBM	meat and bone meal
MD	mule deer
min	minute
mL	millilitre
mM	millimolar
MW	molecular weight marker
NaCl	sodium chloride
NaOH	sodium hydroxide
NBH	normal brain homogenate
NMR	nuclear magnetic resonance
OD	optical density
OLF	Ottawa Laboratory Fallowfield
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween
PCR	polymerase chain reaction
pg	picogram
PI	post inoculation
PK	proteinase K
pM	picomolar
PMCA	protein misfolding cyclic amplification
poly A	polyadenylic acid
Prnp	prion protein gene
Prnd	dopple protein gene
PrP	prion protein
PrP^{C}	cellular prion protein
PrP^{CWD}	infectious prion protein of chronic wasting disease
PrP^{Sc}	infectious prion protein
PrP^{Res}	resistant prion protein (PrP^{Sc}) not destroyed by PK digestion
PVDF	polyvinylidene difluoride
QUIC	quaking-induced conversion assay
Rd	round
RLN	retropharyngeal lymph node
RNA	ribonucleic acid

Sc237	scrapie hamster line
sCJD	sporadic Creutzfeldt-Jakob disease
SDS	sodium dodecyl sulfate
sec	second
sPMCA	serial PMCA
Sprn	Shadoo protein gene
Tg	transgenic
Tgcer	cervidized transgenic mice
TME	mink spongiform encephalopathy
TSE	transmissible spongiform encephalopathy
UK	United Kingdom
USA	United States of America
vCJD	variant Creutzfeldt-Jakob disease
V	volts
v/v	volume/volume
WB	Western blot
WTD	white-tailed deer
w/v	weight/volume
µg	microgram
µL	microlitre

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Prion diseases, or transmissible spongiform encephalopathies, (TSEs) are a family of rare progressive neurodegenerative disorders that affect both humans and animals. TSEs are distinguished by long incubation periods with consistent symptoms becoming apparent only near the terminal stages. Characteristic spongiform changes are associated with neuronal loss and a failure to induce an inflammatory response (Budka *et al.*, 1995). These features pose many challenges for diagnosis, prevention and treatment compared to other transmissible diseases. TSEs are thought to be the result of the accumulation of a misfolded, beta-sheet rich conformer (PrP^{Sc}) of the cellular prion protein (PrP^{C}) (Prusiner, 1998). Accumulation of PrP^{Sc} interferes with normal biological function leading to the creation of vacuoles throughout the nervous tissue, resulting in a sponge-like appearance and the characteristic signs and symptoms of the disease. Misfolded prions are pathogenic agents that are transmissible and are able to induce abnormal folding of specific normal cellular prion proteins. Prion diseases are usually rapidly progressive and always fatal. The function of normal prion proteins has yet to be conclusively identified.

1.1 Background: the prion hypothesis

The infectious nature of prion diseases was first reported in 1939 following the accidental transmission of scrapie into sheep (Cullie & Chelle, 1939). Inoculation against a common virus with a formalin extract of tissue unknowingly derived from a scrapie-infected animal infected nearly 10% of the flock. Following this observation, scrapie was subsequently transmitted experimentally

to sheep (Gordon, 1946) and mice (Chandler, 1961). Bjorn Sigurdsson proposed the term “slow virus” in 1954 to explain the lengthy incubation period associated with scrapie (Sigurdsson, 1954). In 1959, while visiting an exhibit on the mysterious and fatal disease, kuru, afflicting the ritually cannibalistic people of the Fore tribe in New Guinea, William Hadlow, a United States Department of Agriculture employee studying the pathology of scrapie, recognized striking similarities between kuru and scrapie, based on the neuropathological changes, neuronal degeneration and intense astrocytosis found in the brain (Hadlow, 2008). The likeness in pathology was made even more similar by the single and multilocular vacuoles in the perikaryon of large neurons. This prompted Hadlow to suggest in a letter to Lancet that kuru, like scrapie, might be a transmissible disease expressed after a long incubation period (Hadlow, 1959). A formal demonstration from Carlton Gajdusek came in 1966 with the transmission of kuru to chimpanzees (Gajdusek *et al.*, 1966). These studies were followed by subsequent studies demonstrating transmission of other human prion diseases, Creutzfeldt-Jakob disease and Gerstmann-Scheinker syndrome, to primates (Gibbs Jr. *et al.*, 1968; Masters *et al.*, 1981).

Once the transmissibility of prion diseases had been established, studies to determine the component of the infectious agent responsible for the diseases were pursued. Alper and colleagues (Alper *et al.*, 1967; Alper, 1993) demonstrated that procedures used to obliterate nucleic acids, such as ionizing radiation and UV did not destroy the infectious agent. This group also reported that the minimum molecular weight to maintain infectivity (~$2x10^5$ Da) was too small to be a virus or other type of microorganism (Alper *et al.*, 1966). Based on these observations,

speculation arose that the infectious agent responsible for these degenerative diseases was different from any known microorganism and might instead be protein-based (Pattison & Jones, 1967; Griffith, 1967). Prusiner and colleagues (Prusiner, 1982) pioneered a set of discoveries that helped define the prion hypothesis; that a misfolded protein is the main and possibly sole component of this unorthodox infectious agent and coined the term prion to refer to this proteinaceous infectious particle. They demonstrated that protease-resistant prion protein (PrP^{Sc}) could be isolated from the infectious material (Bolton *et al.*, 1982), showing that PrP^{Sc} and infectivity co-purified, and that the protein concentration was proportional to the infectivity titer (Gabizon *et al.*, 1988). Infectivity was retained in highly purified preparations of PrP^{Sc}, in which no other protein was detectable and infectivity was reduced by agents that destroy protein structure and by anti-PrP antibodies (Gabizon *et al.*, 1988). The gene encoding PrP (*Prnp*) was soon discovered (Oesch *et al.*, 1985) and the corresponding mRNA was found to be the product of a single host gene expressed mainly in the brain, without exhibiting expression level differences in healthy or infected animals (Chesebro *et al.*, 1985). These findings indicated that the prion protein exists as both a normal cellular protein (PrP^{C}) and a pathological isoform (PrP^{Sc}) (Basler *et al.*, 1986). Over-expression of mutant *Prnp* in mice produced a transmissible neurodegenerative disease similar to prion disease (Hsiao *et al.*, 1990) and mice lacking the *Prnp* gene were resistant to scrapie infection, developing neither clinical signs of the disease, nor allowing propagation of the infectious agent (Bueler *et al.*, 1993). Cell-free conversion assays also provided support to the prion hypothesis by demonstrating that the conversion of purified PrP^{C} mixed

with stochiometric amounts of purified PrP^{Sc} could produce a low yield of newly synthesized PrP^{Sc} under non-physiological conditions. In 2001, a novel *in vitro* system for replication of prions, termed Protein Misfolding Cyclic Amplification (PMCA), was developed where the newly generated material maintained all the biological, biochemical and structural characteristics of *in vivo* generated prions (Saborio *et al.*, 2001). These findings all support the concept of the prion hypothesis in that newly generated PrP^{Sc} triggers further misfolding to produce an auto-catalytic process of prion replication allowing prions to propagate indefinitely, and leading to the pathology associated with prion diseases (Soto, 2011).

The demonstration that several other proteins in a variety of organisms, such as yeast and fungi, use a protein-based propagation mechanism to transmit biological information also strengthened the prion hypothesis (Wickner, 1994; Halfmann *et al.*, 2010). In yeast, prions do not kill the cells harboring them, nor do they cause disease; rather, they are inherited by cellular division and usually produce new metabolic phenotypes (Soto, 2011). However, like their mammalian counterparts, yeast prions influence other molecules of the same protein to adopt the misfolded form (Soto, 2011). The efficient conversion of a protein function by transmission of an alternate folding state could provide an excellent way in which to modulate the activity of proteins without the need for genetic changes (Halfmann *et al.*, 2010).

Several aspects of PrP^{Sc} infectivity still remain unclear however, and in particular, the detailed structure of the infectious agent and whether cofactors are required for prion replication. The existence of a host-encoded conversion factor

was first suspected from experiments with transgenic mice expressing chimeric prion proteins from two different species (Telling *et al.*, 1995). Based on the findings that supported the hypothesis that a species-specific macromolecule may function as a molecular chaperone in the formation of PrP^{Sc}, the term "protein X" was used to describe this unidentified entity (Telling *et al.*, 1995).. Further evidence came from PMCA studies. Purified hamster PrP^{C} was not converted when mixed with highly purified PrP^{Sc}; however, conversion was restored when the complete brain homogenate was added to the sample (Saborío *et al.*, 1999; Deleault *et al.*, 2005). These results suggested that unknown factors present in brain homogenate are essential for prion conversion. While still unidentified, there are at least 5 possible roles by which co-factors may participate in prion replication (Soto, 2011): i) the co-factor might integrate into the infectious agent, alter PrP^{Sc} folding and provide biological information to the infectivity process, perhaps by determining strain characteristics; ii) the co-factor might act as an essential catalyst for prion replication, perhaps by interacting with PrP^{C}, altering its folding and permitting its interaction with PrP^{Sc}; iii) through binding and integration into the PrP^{Sc} polymer, the co-factor might help stabilize the conformation of PrP^{Sc}; iv) the cofactor might participate in the key process of fragmenting PrP^{Sc} polymers to produce smaller structures and multiplying the number of seeds to allow the continuation of prion replication; and v) the co-factor might bind to PrP^{Sc}, increasing its biological stability, reducing its clearance *in vivo* and increasing its chances to reach target organs. Although it is clear that PrP^{Sc} is the main informational molecule in the infectious agent, it

remains possible that a co-factor is required, perhaps to achieve efficient propagation in the *in vivo* setting (Soto, 2011).

1.1.1 Prion protein structure and function

A chromosomal gene encodes the prion protein (PrP) and is denoted *Prnp*. It is a member of the *Prn* gene family that also includes *Prnd*, encoding the doppel protein (Moore *et al.*, 1999) and *Sprn*, encoding shadoo (Watts & Westaway, 2007). In Syrian hamsters, PrP^{C} and PrP^{Sc} are both 209-residue proteins with two glycosolation sites and a glycosylphosphatidyl inositol (GPI) anchor. No posttranslational modifications to the primary structure differentiate PrP^{C} from PrP^{Sc} (Stahl *et al.*, 1993). Limited protease digestion of PrP^{Sc} often produces a smaller, protease resistant molecule of approximately 142 amino acids, referred to as PrP 27-30 or PrP^{Res}. Under the same conditions, PrP^{C} and some forms of PrP^{Sc} are completely hydrolyzed by protease digestion (Colby & Prusiner, 2011). Furthermore, PrP^{Sc} from different species or prion strains may show different degrees of protease resistance (Colby *et al.*, 2010). PrP^{Sc} is insoluble and forms aggregates with some degree of disorder, but no successful crystallization or solution-based nuclear magnetic resonance (NMR) structures of the PrP^{Sc} have been reported. Investigations using solid-state NMR have been limited by the ability to produce labeled PrP^{Sc} and by the molecular size of PrP^{C}. Electron crystallography coupled with computational modeling identified trimeric, left-handed parallel beta-helices as the most likely substructure for PrP^{Sc} (Govaerts *et al.*, 2004).

Nuclear magnetic resonance structures of recombinant PrP^{C} from many different species have been solved over the past 15 years, representing the best estimate of the structure of PrP^{C}. In addition to a variable octarepeat region and a single disulfide bond, all reveal a three alpha-helix bundle protein with two short anti-parallel beta-strands, (Zahn *et al.*, 2000). These well-folded structural elements are composed of the carboxyl terminus of the protein; the amino-terminal domain is highly flexible and lacks identifiable secondary structure under the experimental conditions employed (Donne *et al.*, 1997). More recently, a crystal structure of PrP^{C} has been obtained, largely in agreement with the NMR structures (Antonyuk *et al.*, 2009). Determining the structural features that differ between PrP^{C} and PrP^{Sc} will likely provide important insight into the pathogenic conversion of PrP^{C} into PrP^{Sc}.

Variations in *Prnp* sequences exist both between species and between individuals within species, which greatly affects susceptibility to prion infection (Colby & Prusiner, 2011). However, the alignment of the protein translated sequences from more than forty mammalian PrP genes shows a striking degree of conservation between the mammalian sequences, suggesting the retention of some important function for PrP^{C} through evolution, but the specific role of this protein has not been identified. Copper binding, which may play a role in the kinetics and pathobiology of prion diseases, has been speculated (Millhauser, 2004). Addition of copper has been shown to assist the conversion of PrP^{C} to a partially protease resistant form (Quaglio *et al.*, 2001) and also to increase PrP^{C} endocytosis, thereby affecting PrP^{C} trafficking (Pauly & Harris, 1998). PrP knockout mice have also been shown to have significantly less copper associated with their

brains than wild type mice, suggesting a strong linkage between PrP and copper (Millhauser, 2004). The similarities and potentially overlapping functions with other members of the *Prn* gene family such as shadoo may eventually aid in further elucidating the true function of PrP (Watts & Westaway, 2007).

1.1.2 Infectivity, persistence and resistance

Misfolded infectious prions are extremely resistant to degradation and show remarkable resilience to decomposition in the environment. Brown and Gajdusek originally demonstrated detection of prion infectivity after exposure to the soil environment (Brown & Gajdusek, 1991). In these studies, scrapie-infected hamster brain homogenate mixed with soil and stored outdoors in buried flowerpots for three years remained infectious. Johnson and colleagues have shown that prions bound to soil minerals, particularly montmorillonite clay, not only remain infectious (C. J. Johnson *et al.*, 2006) but actually become more infectious than unbound prions (C. J. Johnson *et al.*, 2007). Increased infectivity could be due to conformational changes in PrP^{Sc}, including changes in aggregation, upon binding (Saunders *et al.*, 2008). The potential for CWD or scrapie transmission by exposure to contaminated soil is possible since cervids and ruminants are known to ingest and inhale large amounts of soil (Beyer *et al.*, 1994). In one report, a scrapie infected sheep-house and pasture were decontaminated and left uninhabited for 16 years later, and when sheep were reintroduced to the premises they subsequently contracted scrapie (Georgsson *et al.*, 2006). In a controlled lab study, Miller *et al.* (2004) demonstrated CWD

transmission to naïve captured mule deer allowed to graze in pastures in which CWD-positive deer resided approximately 2 years previously (Miller *et al.*, 2004). Epidemiological modeling also suggests that indirect, environmental routes of transmission were responsible for two CWD outbreaks in captive mule deer (Miller *et al.*, 2006). To persist in soil environments, prion stability is required to withstand extreme conditions including freeze thaw cycles, extracellular enzymes from fungi and bacteria, UV radiation, digestion by soil microbes, and abiotic transformation through mineral phases (Taylor, 2000).

Prions have also been shown to adsorb strongly to metal, which poses significant problems in medical and research fields. The ability of infectious prions to withstand radiation, proteases, chemical disinfectants, preservation in fixatives, autoclaving and even ashing (360°C) (C. J. Johnson *et al.*, 2009), means the universal precautions employed by hospitals and medical research labs are often not extensive enough to eliminate the risk of exposure. Neurosurgical equipment used on a pre-clinical undiagnosed CJD patient led to the subsequent transmission of disease to two other patients (Bernoulli *et al.*, 1977). This was confirmed by the implantation of the tip of the instrument into the brain of a chimpanzee, proving that the infectious agent had remained intact for several years through numerous disinfection and sterilization processes (Gibbs Jr. *et al.*, 1994). The increased rate of sCJD has been speculated to be related to the increased number of surgical procedures being performed (Ward & Knight, 2008), yet harsh decontamination procedures outlined for prion removal or inactivation are often too damaging and impractical for most surgical instruments (Sutton *et al.*, 2006). For these reasons, prion research is restricted to laboratories

where dedicated prion handling facilities exist, in order to contain these agents. Equipment, instruments and waste material generated in these facilities can then be exposed to extreme conditions such as strong sodium hypochlorite solutions, hot solutions of sodium hydroxide or autoclaving at 134°C for 60 minutes, recognized to inactivate infectious prions (Taylor, 2003).

1.1.3 Human prion diseases

Prion diseases can occur sporadically, genetically or through transmission. In humans, sporadic and inheritable forms of the disease occur more frequent than infectious forms, with sporadic CJD (sCJD) accounting for approximately 85% of known cases (R. T. Johnson, 2005). Other than the spontaneous misfolding of PrP^{C} into PrP^{Sc} (Prusiner, 1998), sCJD has no known cause, but hypotheses include somatic mutation of the *Prnp* gene, undetected horizontal transmission from unidentified sources, and infrequent amplification of low levels of PrP^{Sc} that are a normal part of protein homeostasis (Colby & Prusiner, 2011). Misfolded prions from sCJD patients are able to cause disease when transferred to experimental animals (Gibbs Jr. *et al.*, 1968). Neuropathological changes such as PrP^{Sc} deposition, astrocytic gliosis and vacuolation, resulting in the sponge-like appearance of the brain, are common features found among the human prion diseases. The precise morphology of these features, however, varies depending on the prion strain and host, as do the regions of the affected brain. Clinical features of human prion disease include rapid progressive dementia, ataxia (loss of muscle coordination), and progressive blindness (Brown *et al.*, 1994).

Genetically-related prion diseases have been described in humans, and include Gerstmann-Sträussler-Scheinker syndrome (GSS), familial CJD (fCJD) and fatal familial insomnia (FFI). These diseases are characterized according to their clinical symptoms, but all result from mutations in the *Prnp* gene. Over forty different mutations in the *Prnp* gene have been shown to cause inherited human prion disease (Colby & Prusiner, 2011). It was the discovery that a proline to leucine mutation at codon 102 of the *Prnp* gene was genetically linked to some GSS pedigrees that allowed for the conclusion that prion diseases can have both genetic and infectious etiologies (Hsiao *et al.*, 1989).

Infectious forms of prion diseases in humans include kuru, iatrogenic CJD (iCJD) and variant CJD (vCJD). Kuru was transmitted by ritualistic cannibalism (Gajdusek, 1977). In ceremonies to immortalize dead relatives, members of the Fore tribe in the highlands of New Guinea would consume the brains from family members who had recently died, unknown to them of suffering from a prion disease (Gajdusek, 1977). Iatrogenic (illness caused by medical examination or treatment) transmissions include prion-tainted human growth hormone (Koch *et al.*, 1985; Gibbs Jr. *et al.*, 1985) and gonadotropin (Cochius *et al.*, 1990), dura mater grafts (Hannah *et al.*, 2001), and transplants of corneas obtained from people who died of CJD (Hogan *et al.*, 2003). New CJD cases have also resulted from neurosurgical procedures using instruments that were ineffectively sterilized, after coming in contact with infectious prions (Bernoulli *et al.*, 1977). Consumption of beef products contaminated with bovine spongiform encephalopathy is thought to be the cause of vCJD, which by August 2013 had resulted in 176 definite or probable cases of vCJD confirmed in the UK, with 51

additional cases in 11 other countries and an additional 2 cases who are still alive (UK and France)[1].

Until recently, all of the vCJD individuals were identified to be homozygous for methionine at codon 129 of the human prion gene, but a single case of a vCJD patient heterozygous at codon 129 has been reported raising the possibility of a second wave of "mad cow" related deaths (Kaski *et al.*, 2009).

1.1.4 Animal prion diseases

Many mammals are vulnerable to infectious prion diseases and display many of the same neuropathological changes including PrP^{Sc} deposition, astrocytic gliosis and vacuolation that are observed in human cases. Clinical features include behavioral changes, ataxia (loss of muscle coordination), hyperexcitability, tremors and progressive blindness (Imran & Mahmood, 2011). Scrapie is found in sheep and goats; bovine spongiform encephalopathy (BSE) in cattle; transmissible mink encephalopathy (TME) in mink; chronic wasting disease (CWD) in deer, elk, moose and caribou; feline spongiform encephalopathy (FSE) in cats; and exotic ungulate encephalopathy in kudu, nyala and oryx (Colby & Prusiner, 2011).

1.1.4.1 Scrapie

Scrapie of sheep has been recognized since 1732 (Jeffrey & González, 2007). To date, no evidence exists linking scrapie to CJD in humans (Colby &

[1] http://www.cjd.ed.ac.uk/documents/worldfigs.pdf (Last viewed on August 27, 2013)

Prusiner, 2011). In typical scrapie, intense pruritus of the skin is the most common symptom usually leading to wool loss by rubbing and scraping (Kimberlin, 1981). The incubation period is 2-5 years and death occurs within 2 weeks to 6 months after clinical onset. PrP^{Sc} has been detected in the nervous system, tonsils, spleen, lymph nodes, nictitating membrane (third eye lid), muscles, placenta, distal ileum and proximal colon. Detection of PrP^{Sc} in the third eye lid is used as a rapid diagnostic test in live animals. PrP^{Sc} infectivity has also been found in excretions and secretions that may contribute to horizontal transmission of scrapie through the environment. Scrapie susceptibility has been reported to be associated with several amino acid polymorphisms in the ovine *Prnp* gene (Goldmann, 2008). A three-codon system based on A136V, R154H and Q171R/H polymorphisms has been developed by using five alleles (ARQ, VRQ, AHQ, ARR, ARH) to describe resistant/susceptible genotypes in sheep. The most resistant genotype is ARR/ARR and the most susceptible genotypes are VRQ/VRQ, VRQ/ARQ, VRQ/ARH and VRQ/ AHQ with the remaining genotypes of intermediary susceptibility. This risk classification system has been used in selective breeding of sheep against scrapie with no considerable negative impacts on production, reproduction or health. Atypical cases of both sheep and goat scrapie have also been described with the major clinical features being ataxia and incoordination whereas pruritus is uncommon (Benestad *et al.*, 2003). Atypical scrapie is also influenced by polymorphisms in the *Prnp* gene and has been reported to occur in animals carrying genotypes conferring resistance to typical scrapie (Dawson *et al.* 2008). It was once thought that control of TSEs could be possible by using knowledge of PrP genetics to breed for resistance, but

the occurrence of atypical scrapie, puts this stance into question (Goldmann, 2008). It now appears that all efforts to breed for classical scrapie resistance may have ultimately lead to an increased risk for the population to have carriers of atypical scrapie (Goldmann, 2008). A search for an explanation for why the susceptibility for both forms of scrapie apparently resides at the opposite spectrum of the known PrP genetics is currently underway. It may reflect the selection by two different agent strains or involve novel PrP or non-PrP genetics (Goldmann, 2008). The prion protein's function is currently unknown, but once discovered may shed light on this mysterious phenomenon and perhaps reveal how the misfolding provides an evolutionary advantage and thus persists through time.

1.1.4.2 Bovine spongiform encephalopathy

In bovine spongiform encephalopathy (BSE), also known as "Mad Cow disease", PrP^{Sc} accumulation and spongiform vacuolization are usually found in the brain. At the terminal stages of the disease, BSE prions may also be detected in spinal cord, retina, ileum, adrenal glands, tonsils, bone marrow, peripheral nerves, dorsal root ganglia, trigeminal ganglion, and thoracic ganglia (Novakofski *et al.*, 2005). BSE infectivity may be observed as early as 2 years post inoculation, with an incubation period from 2 to 8 years. Studies have found no evidence of BSE prions in milk, semen or embryos and there is little to no evidence of horizontal transmission. However the offspring of infected animals have shown an increased risk for disease development (Imran & Mahmood, 2011). Atypical BSE strains (H-type and L-type) have also been described with the main difference

being the presence of amyloid plaques in the brain unlike classical BSE (Novakofski *et al.*, 2005).

BSE first appeared in the mid 1980s in the UK. It was estimated that at its peak, nearly one million cattle were infected, reaching epidemic proportions with 1000 new cases occurring per week in 1992 (R. M. Anderson *et al.*, 1996). The practice of using meat and bone meal (MBM) contaminated with infectious mammalian pathogenic prions in cattle feed is considered the most likely cause of the BSE epidemic (Imran & Mahmood, 2011). MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high protein nutritional supplement. In the late 1970s, the hydrocarbon-solvent extraction method used in rendering of offal began to be abandoned to produce an MBM with a much higher fat content (Müller *et al.*, 2007). It is now thought that this change allowed scrapie prions from sheep or low levels of bovine prions generated sporadically to survive the rendering process, resulting in the widespread infection of cattle (Colby & Prusiner, 2011). A ban on the use of MBM in the ruminants' feed ultimately resulted in a progressive decline of the epidemic (Ducrot *et al*., 2008).

1.1.4.3 Chronic wasting disease

Until recently, chronic wasting disease (CWD), a natural prion disease of cervids, was believed to infect only three members of the Cervidae family: mule deer (*Odocoileus hemionus*), white tailed deer (*Odocoileus virginianus*) and Rocky mountain elk (*Cervus elaphus nelsoni*) (Williams, 2005). In 2006, a report indicated that Shira's moose (*Alces alces shirasi*) could be orally infected with brain homogenate from CWD infected mule deer, and shortly thereafter a natural

case was discovered bringing the total number of susceptible species to four (Kreeger *et al.*, 2006; Baeten *et al.*, 2007). This situation may be repeated in reindeer (*Rangifer tarandus tarandus*) as experimental studies have shown susceptibility of this species to CWD (Mitchell *et al.*, 2012). Due to the high level of contagion, CWD is the only known prion disease to affect both free-ranging non-domesticated animals as well as captive animals. Until recently CWD was only found in North America, but unfortunately it has now been exported to South Korea (Kim *et al.*, 2005). North America is currently experiencing an epidemic of CWD. Although certain primate species have been shown to be susceptible to CWD by intracerebral and oral feeding studies (Marsh *et al.*, 2005; B. Race *et al.*, 2009) no evidence suggests that CWD currently represents a threat to humans (Saunders *et al.*, 2012).

1.1.4.3.1 Discovery of CWD

In the late 1960s, biologists conducting physiological and nutritional studies on mule deer noticed that their adult research animals often died of a syndrome of progressive weight loss and behavioral changes. It was initially thought to be associated with stress due to captivity, nutritional deficiencies or exposure to toxins (Williams & Young, 1980). It was not until 1978 that this chronic wasting condition was identified as a TSE by histological examination of the brains of affected deer from wildlife research facilities in Colorado and Wyoming (Williams & Young, 1980). Within a few years a similar TSE was diagnosed in Rocky Mountain elk and black-tailed deer (*Odocoileus hemionus colombianus*) held in the same facilities where the mule deer had previously been diagnosed

with the condition. Epidemic modeling suggested that CWD might have been present among free ranging animals in some portions of the disease-endemic area several decades before it was initially recognized (Miller *et al.*, 2000). At this time, CWD was also found in a zoo in Ontario Canada in several mule deer that had originated from a Colorado zoo (Kahn *et al.*, 2004).

Rocky Mountain elk, also known as wapiti, were the first commercially farmed animals to be detected with CWD (Kahn *et al.*, 2004). These animals were found on a game farm in Saskatchewan, Canada in 1996, but subsequent investigations indicated that the most likely source was captive elk imported from South Dakota prior to 1989 (Williams, 2005). This led to the detection of several other infected game farms in Saskatchewan as well as across the border in South Dakota (Kahn *et al.*, 2004). Implementation of surveillance programs at the provincial, state, and federal levels for captive cervids resulted in the detection of CWD in ten states in the United States (U.S.) and two provinces in Canada. To date, isolated, non-contiguous clusters of CWD infected cervids have been located in 15 states, from New York and West Virginia in the east, and as far west as Utah, Saskatchewan and Alberta (Saunders *et al.*, 2012). Prevalence can reach as high as 30% for free ranging deer and over 90% in facilities where the disease has been endemic for over 2 years (Wild *et al.*, 2011). The movement of captive elk for commercial farming has also led to exportation of CWD to South Korea, the only known country outside of Canada and the U.S. to have the disease (Kim *et al.*, 2005), leading to minimal surveillance on an international level with the exception of a CWD surveillance program in Germany (Schettler *et al.*, 2006).

1.1.4.3.2 Clinical signs

The most obvious sign of the disease, as suggested by its name, is the loss of body condition that occurs in the later stages and progresses to a state of emaciation if allowed to run its course. The duration of the disease from the onset of illness can last from a few months to over a year with the majority of captive cervid cases occurring between the ages of 2 and 7 years old. CWD has been observed in cervids as young as 17 months old and as old as 15 years of age (Williams, 2002). The duration of the disease is assumed to be shorter in free-ranging cervids as compared to domestic cervids. As disease progresses, difficulties in foraging for food and water and avoiding predators results in greater vulnerability to an earlier death (Williams *et al.*, 2002).

Like most diseases, clinical signs can vary among individuals. Consistent signs are only observed near the terminal stages and some signs are never exhibited at all. It is estimated that more than 97% of the CWD cases in the wild show no clinical signs making identification difficult (Williams, 2005). Descriptions are mainly based on captive animals, with the progression in free ranging animals being less documented. Because the earliest signs are so subtle, more so in elk than deer, caretakers who are familiar with the animal's individual nature are usually the first to notice things such as isolation from the herd and depression. Changes in the day-to-day interactions within the herd and towards people are usually the first signs. Walking repetitive patterns in the pen, abnormal drowsiness or hyperexcitability when restrained can follow (Miller & Wild, 2004). Later stage clinical signs may include hypersalivation (sialorrhea) due to difficulty swallowing which can be the result of a dilated, spastic or flaccid

oesophagus. Excessive thirst (polydipsia) and consequently excessive urination (polyuria) follow. Stumbling and incoordination (ataxia), head tremors, abnormal tongue movements, regurgitation and tooth grinding (odontoprisis) may also be observed (Williams & Miller, 2002), but the itching and scratching (pruritus) associated with scapie is not a characteristic of CWD (Williams & Young, 1992). Terminal physiological and behavioral changes may also involve a sudden loss of consciousness due to a rapid decrease in blood pressure (syncope), periods of lack of awareness with a fixed stare and an altered stance with a lowered head (Williams, 2005). Aspiration pneumonia can occur at any time following the loss of motor control over swallowing and may be responsible for rapid deaths in the absence of any other signs (Williams & Miller, 2002). Other general finding through autopsies of infected cervids have found bronchopneumonia from fluid in the lungs, frothy or watery rumen contents containing sand, abomasal or omasal ulcers, serious atrophy of bone marrow and pericardial fat, enlarged adrenal glands and muscle atrophy (Spraker *et al.*, 1997).

1.1.4.3.3 PrP distribution and target organs

The CWD infectious agent (PrP^{CWD}) is rapidly deposited throughout the central nervous system and extraneural tissues, with the nucleus of the vagus nerve being affected most consistently and severely by the vacuole producing spongiform changes in all host species. Through immunohistochemistry (IHC), accumulation of PrP^{CWD} has been found in the brain, spinal cord, nerves and ganglia of the peripheral nervous system as well as the endocrine and lymphoid tissues and can occur without the presence of spongiform lesions or clinical signs

making antemortum testing difficult and unreliable (Williams, 2005). Target areas for severe spongiform change include olfactory bulb and stria, septal nuclei, thalamus, supraoptic and paraventricular nuclei, tegmental nuclei, and within the medulla oblongata, neurons of the reticular formation, as well as the nuclei from the hypoglossal vagal, medial and lateral cuneatus, and spinal tract of the trigeminal nerve (Williams, 2005). Lesions in the brain are similar to the ones found in BSE and scrapie consisting of perikaryonic neuronal vacuoles, microcavitation of gray matter, astrogliosis, neuronal degeneration and loss, and infectious PrP positively labeled prion deposits and plaques (Williams, 2005). Elk are known to have more severe lesions in the thalamus and some white matter areas as compared to deer but conversely congo red bifringent, PAS-positive amyloid plaques have been found in deer but not elk (Williams & Young, 1993).

In as short as 6 weeks post oral inoculation (PI), deposits of the abnormal prion protein (PrP^{CWD}) can be found in alimentary tract-associated lymphoid tissues like the retropharyngeal lymph nodes of mule deer (Sigurdson *et al.*, 1999) and in tonsils as much as 20 months prior to death from naturally occurring CWD (Wild *et al.*, 2002). Because the infectious prion protein is typically disseminated throughout the lymphoid system before the central nervous system, tonsil biopsy can be used to diagnose CWD before death (antemortem), but unfortunately not all follicles are necessarily positive and not all CWD cases have a lymphoid phase of infection so a negative tonsil biopsy cannot rule out a CWD diagnosis (Sigurdson & Aguzzi, 2007). This is particularly true in elk, where one study found that 28 out of 226 CWD positive elk had no detectable infectious PrP in their lymphoid systems at all (Spraker *et al.*, 2004). It has been shown that the

presence of infectious prions in retropharyngeal lymph nodes and in rectal biopsies is consistent later into the disease. Though less accurate earlier on, rectal lymphoid biopsies may allow for a possible large-scale surveillance and management strategy based on the ease of sampling and rapid test analysis (Williams, 2005).

By three months oral PI, PrP^{CWD} has typically spread throughout the lymphoid tissues, tonsils, Peyer's patches and ileocaecal lymph nodes (Sigurdson *et al.*, 1999) and by nine months can be found in the myenteric and submucosal plexi in the gastrointestinal tract and in the vagus nerve (Sigurdson, 2008). This supports the hypothesis that autonomic innervation, particularly of enteric tissues, plays a role in the spread of PrP^{CWD} from peripheral tissues to the central nervous system (Sigurdson, 2008). By 16 months oral PI, PrP^{CWD} is detectable throughout the spinal column and brain leading to the characteristic spongiform encephalopathy associated with the prion diseases (Sigurdson, 2008).

PrP^{CWD} has also been found in other non-CNS tissues such as pancreatic islets, spleen, adrenal gland, antler velvet, skeletal muscle and for the first time for any natural TSE, cardiac muscle (Jewell *et al.*, 2006). The presence of PrP^{CWD} in saliva, blood, urine and feces has also provided evidence of possible routes of transmission through the environment to explain the highly contagious spread of the disease. For instance, orally inoculated deer have been shown to shed infectious prions in saliva and feces 6-11 months or more before the onset of clinical signs (Mathiason *et al.*, 2009; Tamguney *et al.*, 2009).

1.1.4.3.4 CWD genetic susceptibility and resistance

Prion protein structure in deer and elk may play a role in disease transmission efficiency. The *Prpn* gene is highly conserved in cervids with only a few amino acid residues differing among the different species and several species-specific polymorphisms exist at certain codons that may influence CWD susceptibility (O'Rourke *et al.*, 1999). One study looking at 1482 mule deer found that when comparing the genotype frequency of CWD positive to CWD negative free-ranging mule deer, there was a 30 times greater chance that the infected animal was 225SS as opposed to 225SF suggesting that serine homozygosity at *Prnp* codon 225 seems to increase the risk for CWD infection (Jewell *et al.*, 2005).

White-tailed deer also have polymorphism at *Prpn* codon 96 (S/G). When the allelic frequencies from CWD-positive and CWD-negative free-ranging Wisconsin white-tailed deer were compared, it was found that polymorphisms at G96S and Q95H were underrepresented in the free-range population suggesting a reduced susceptibility to CWD (C. Johnson *et al.*, 2006). In experiments by Meade-White *et al.* (Meade-White *et al.*, 2007), when transgenic mice expressing cervid PrP of either allelic variant (denoted as tg[CerPrP96G] and tg[CerPrP96S]) were exposed to mule deer CWD, tg(CerPrP96G) mice developed disease 160 days post-infection.whereas tg(CerPrP96S) mice were completely resistant, with no clinical disease or PrP^{Sc} deposition even at 600 days post-infection.

Elk have a polymorphism at codon 132 (M/L) of *Prpn*, corresponding to polymorphism 129 (M/V) in humans. Not only is this codon important for CJD disease susceptibility in humans and strain typing, but this codon also seems to be

influential for elk prion susceptibility (Wadsworth & Collinge, 2007). Among free-ranging and captive elk, animals expressing the codons 132MM and 132ML were found to be statistically overrepresented among elk with CWD when compared to uninfected controls (O'Rourke *et al.,* 1999). Elk with the codon 132LL when experimentally infected with CWD, developed clinical disease by 59-64 months post-inoculation, whereas 132MM and 132ML elk cohorts came down with CWD at 23 and 40 months post inoculation. Therefore the 132LL homozygous and 132ML heterozygous elk had a 2-3-fold delay in the infection kinetics, indicating that the 132 polymorphism may influence prion conversion in elk (O'Rourke *et al.,* 2007).

NMR analysis of recombinant elk PrP^{C}, has shown an extremely well defined loop connecting the second alpha helix and beta sheet (amino acids 166-175). In the homologous regions of the human and bovine PrP, this is flexibly disordered. This non-conservative species variation of the loop has produced great interest possibly leading to structural insights into the species barrier phenomenon (Calzolai *et al.*, 2000).

Another interesting feature of deer and elk prion genetics from an evolutionary perspective is the presence of a pseudogene described in both mule and white-tailed deer (WTD) (Brayton *et al.*, 2004; O'Rourke *et al.*, 2004). The pseudogene is thought to be a retrotransposon (genetic elements that can amplify themselves in a genome and are ubiquitous components of the DNA of many eukaryotic organisms), since it lacks introns and it is flanked by direct repeats (O'Rourke *et al.*, 2004). In WTD, the pseudogene encodes five or six octapeptide repeats. At residue 138, the *Prpn* functional and pseudogene diverge, encoding a

serine or asparagine, respectively. Neither Old World Rocky Mountain elk nor New World moose possess the pseudogene, possibly indicating that the *Prpn* pseudogene arose after evolutionary radiation of *Odocoileus* in the New World (O'Rourke *et al.*, 2004).

1.1.4.3.5 Evolutionary origin theories

The origins of CWD are unknown, but the disease agent may have evolved out of scrapie from domesticated sheep. Arguments supporting scrapie as a possible source of CWD include: (i) the similarities in glycoform patterns on Western blots of diseased-associated prion protein derived from CWD affected deer and elk and sheep scrapie (R. E. Race *et al.*, 2002); (ii) the moderate ability of prions derived from CWD affected deer and elk to convert cellular prion protein from sheep to the abnormal isoform and conversely, for prions derived from scrapie-affected sheep to convert cellular prion protein from deer or elk to abnormal isoforms (Raymond *et al.*, 2000) and (iii) the occurrence of CWD like clinical disease and lesions in elk inoculated intercranially (i.c.) with scrapie agent (Raymond *et al.* 2000). Arguments against scrapie as a source of CWD, though, include: (i) the apparently limited distribution of CWD compared to scrapie with its geographic overlap with cervid species worldwide (Williams & Young, 1992); (ii) the relatively long incubation seen after i.c. inoculation of CWD into goats and cattle (Hamir *et al.*, 2005); (iii) that it does not appear to be transmissible to raccoons as is scrapie (Hamir *et al.*, 2003) and (iv) the apparent strain differences between CWD and other recognized prion diseases (Raymond *et al.*, 2000).

The epidemiology of CWD is most comparable with a single strain that originated in mule deer and then infected elk and white-tailed deer (Williams & Young, 1992). CWD was first recognized as a clinical syndrome in mule deer many years before it was detected in elk housed in facilities with CWD-affected mule deer (Williams & Young, 1992). The first recognition of CWD in WTD was in free-ranging deer overlapping in distribution with CWD-affected mule deer and elk by retrospective examination of tissues when immunohistochemistry for PrP^{CWD} became available (Williams & Miller, 2002).

CWD may also represent a new or re-emerging spontaneous, naturally occurring spongiform encephalopathy of cervids that may have had the opportunity to reach a tipping point to become an epidemic due to the absence of large predators such as wolves and mountain lions (Wild *et al.*, 2011). Wolves and mountain lions have been absent from Colorado since the 1940s when they were completely exterminated by federally funded bounty hunters (Miller *et al.*, 2000). It is generally believed that the original emergence of CWD took place in Colorado and by removing certain predators an environmental imbalance may have been created allowing for the establishment of CWD to take place (Wild *et al.*, 2011). The relatively limited geographic distribution of CWD further supports this hypothesis (Williams *et al.*, 2002). The prolonged clinical course and type of clinical abnormalities associated with CWD make it the prototypic disease for selection by predators with subtle changes in behavior and body condition that progress over weeks or months to overt signs of end-stage disease. Loss of attentiveness and cognitive function likely account for the marked increase in risk for vehicle collision compared to hunter-harvested deer (Krumm *et al.*, 2005). It

follows that infected cervids would also be less attentive to predators and possibly explains a nearly four fold greater relative risk of infected deer succumbing to mountain lion predation (Miller *et al.*, 2008). Consumption by predators would also be expected to reduce the contribution of carcass material to the overall pool of environmental infectivity. Passage through the alimentary tract of wolves likely degrades infectivity of tissues (Wild *et al.*, 2011). In sheep, *in vitro* incubation of a dilute scrapie brain inoculum with alimentary tract fluids resulted in almost complete degradation of PrP^{Res} (Jeffrey *et al.*, 2006).

1.1.4.3.6 Transmission

While CWD is found naturally in the four previously mentioned species, it has been transmitted, into cattle (Hamir *et al.*, 2001), goats (Williams & Young, 1992) and domestic sheep (Hamir *et al.*, 2006), but only by intracerebral inoculation and with relative inefficiency suggesting a strong species barrier. Laboratory animals such as mice (Browning *et al.*, 2004), ferrets (Sigurdson *et al.*, 2008), mink (Harrington *et al.*, 2008), voles (Heisey *et al.*, 2010) and squirrel monkeys (Marsh *et al.*, 2005) have also been found susceptible to CWD by intracerebral inoculation as well as hamsters, following passage through ferrets (Bartz *et al.*, 1998). Deer-to-deer and elk-to-elk transmission is readily performed by oral inoculation, as is elk-to-mule deer and white-tailed deer and mule deer to elk and white tailed deer (Williams & Young, 1992).

This ease of oral transmission among cervids may account for some form of direct or indirect transmission that sustains the current epidemic. The natural incubation period of CWD is not known but deer and elk under two years of age

have tested positive suggesting a minimum incubation period of approximately 1.5 years, a value which correlates with experimentally infected mule deer (Williams & Miller, 2002). Unlike the human TSEs, or BSE, environmental contamination from live infected animals is probably the primary source of new infections and geographic spread, with an oral and/or nasal mucous membrane entry route the most likely path into the animal (Denkers *et al.*, 2011). The vector of entry and the exit route still remain a mystery though. Miller *et al.* (2004) have shown CWD is transmitted to captive deer allowed to graze in pastures in which CWD-positive deer resided approximately 2 years previously and also in fields containing naturally decaying CWD-infected carcasses. Unlike scrapie, transmission from mother to offspring does not seem to play a significant role (Miller & Williams, 2003). Infectivity and PrP^{CWD} deposition have not been found in the placenta of either deer or elk (Williams, 2005). This is in contrast to scrapie, in which high levels of infectivity reside in the placenta, and epidemiologic investigations have found that transmission at the time of lambing appears to be important in maintaining scrapie in flocks (Andréoletti *et al.*, 2002). These factors all suggest a more horizontal form of transmission for CWD.

Because CWD-infected tonsils have an abundance of infectious prions they may serve as a source for prion shedding into saliva and may point towards common water sources and shared salt licks, as well as licking and grooming behavior as possible routes of transmission (O'Rourke *et al.*, 2003; Sigurdson & Aguzzi, 2007). Nichols *et al.* (2009) were able to detect protease-resistant cervid prion protein in environmental water from a CWD-endemic area and Mathiason *et al.* (2006) demonstrated infectious prions could be transmitted by an oral route

from CWD positive deer to CWD-naïve deer in saliva and in blood by transfusion, although the experiment did not reflect natural conditions. Large volumes (50 mL) of saliva administered three times in three consecutive days and blood from terminally ill animals containing high levels of infectious prions were used for the transfusions. This study does, however, reinforce the notion that saliva can act as a vehicle of transmission. In addition, since blood has been shown to contain prion infectivity, arguably, no tissue from CWD-infected cervids can be considered free of prion infectivity. It has also been suggested that there is the potential for hematogenous spread of CWD through biting or blood-sucking insects (Mathiason *et al.*, 2006).

While saliva was shown to transmit prions to naïve deer within 12 months (Mathiason *et al.*, 2006), the same study found that naïve deer inoculated with urine and feces from CWD positive deer had no detectable PrP^{CWD} for up to 18 months PI, suggesting that urine and fecal matter may not be important transmission vectors. The study did however point to several reasons why this negative finding should be viewed cautiously. The two deer in the urine/feces group expressed the G/S polymorphism at PrP codon 96, which has been shown to confer longer incubation times (>18 months) even for high titre inocula prepared from brain homogenate. That, in conjunction with the possible premature termination of the animals before they showed any clinical signs and the small sample size (N=2), lead the authors to suggest these results were inconclusive and further study was needed regarding urine and feces as potential vectors.

Other controversies over urine as a potential vehicle of transmission have occurred. While previous studies have reported low levels of PrP^{Sc} in urine (Kariv-Inbal *et al.*, 2006), conflicting reports have demonstrated that the protease resistant band detected in the Western blots of diseased animals, assumed to be PrP^{Sc}, was actually an artifact produced by a cross reaction of the anti-mouse IgG antibody with IgG light chains and possibly heavy fragments found in the urine and unlikely to be PrP^{Sc} (Head *et al.*, 2005). There has also been debate regarding the methods used to collect the urine. Metabolic cages fail to separate urine from feces and allow for possible contamination of the urine from prions found in the fecal matter (Safar *et al.*, 2008).

Inflammation may also play a key role in prions being present in urine and not reflect a consistent natural pattern. Prion infected mice with follicular inflammation in the kidneys direct prions to accumulate within lymphoid follicles leading to prion excretion into the urine in the same way that follicular mastitis in natural scrapie sheep results in prion accumulation in the mammary glands (Ligios *et al.*, 2005), which is then shed into the milk (Lacroux *et al.*, 2008) and has recently been shown to transmit scrapie to nursing lambs (Konold *et al.*, 2008).

Using source deer with nephritis, Haley *et al.* (2009a) recently demonstrated for the first time infectious CWD prions in the urine and saliva of cervids by transgenic mouse assay and PMCA. By incorporating more sensitive detection systems in conjunction with an intercranial inoculation route, they were able to measure lower levels of prion infectivity than previous studies had allowed. The intercranial bioassay using transgenic mice also permitted extended incubation

periods and a greater number of test animals to solve the criticisms of Mathiason *et al.* (2006). They found only two of the nine *tg1536* mice inoculated with concentrated urine from CWD positive white-tailed deer tested positive for prion infection as confirmed by western blot and immunohistochemistry, with a third positive animal later identified by PMCA. The mice receiving saliva inoculations tested positive for eight out of the nine mice suggesting much higher levels of infectious prions in the saliva and possibly an uneven distribution of infectious prions moities in the urine inocula due to the wide range in survival times.

Safar *et al.* (2008) successfully showed transmission and detection of PrP^{Sc} in feces using hamsters but also used extremely high oral inoculums. Uninoculated hamsters were cohabited with or exposed to the bedding of the infected hamsters. Incubation times of ~140 days and a rate of prion infection of 80%-100% among the exposed animals suggested transmission by feces via coprophagy (ingestion of feces). Hamsters were fed half of the brain from a Sc237-infected Syrian hamster relating to about 10 $\log_{10}$ ID_{50} per dose. The feces in the first week were measured to have titers of 6.6 $\log_{10}$ ID_{50}/g and then decreased to lower levels of ~2.3 $\log_{10}$ ID_{50}/g throughout the asymptomatic phase of the incubation period, most likely due to the shedding of prions from the infected Peyers patches. A triphasic pattern emerged. Uninfected hamsters that cohabitated within hours of an infected hamster's inoculation, developed disease quickly, most likely due to the exposure of high doses of infectious prions that had been rapidly excreted in the feces. Hamsters that had cohabitated 24-48 hours after oral exposure developed disease more slowly (192-254 days), reflecting exposure to lower doses, but animals exposed 7-14 days after exposure showed

relatively short incubation times again (140-165 days). These shorter times are thought to be the result of exposure to infectious prions that had been given enough time to replicate in the intestinal tract and were being shed in the feces (Bosque & Tyler, 2008). This experiment desmonstrated that lateral transmission with feces as a vehicle is possible but critics point out that extrapolating the findings from experimental animal models to natural infection in herds of wild animals remains problematic. The distribution of prions in the host is influenced by both the species of the infected host and by the prions themselves in the form of strains. The strain concept creates problems for animal models. Strains are thought to be a result of subtle changes in conformation that can lead to distinct differences in the clinical, histological, biochemical, and physical outcomes, even when propagated in genetically identical hosts. In the afore-mentioned experiment (Safar *et al.* 2008), mice were also used and intracerebrally inoculated with irradiated feces from mice orally infected with scrapie 237. These mice were compared with mice intracerebrally inoculated with the same prion strain that had not been passed through the gut and excreted. There was immense variation in the distribution and intensities of the neuropathological changes found in the pontine base and cerebellum. Since the mice that were inoculated with prion-free irradiated feces showed no acute toxic symptoms, the findings suggest that the natural and pathogenic properties of the infectious agent may be affected by the route of prion shedding from the infected host or that the presence of feces in the inoculum creates a shift in the strain characteristics. Based on previous examples of strain diversity, it is likely that the distribution and shedding of CWD prions in

cervids differs from Sc237 in hamsters so the findings in inoculated lab animals to natural infections remain questionable (Bosque & Tyler, 2008).

Tamgüney *et al.* (2009) were able to show that asymptomatic CWD infected mule deer excrete infectious prions in their feces long before they develop clinical signs of the disease. Intracerebral inoculation of irradiated deer feces into transgenic mice over expressing cervid prion protein revealed infectivity in 14 of 15 fecal samples collected from five deer at 7-11 months before the onset of neurological disease. Although the prion concentrations in deer feces were much lower than in brain tissue from the same deer collected at the end of the disease, the estimated total infectious dose excreted in feces by an infected deer over the disease course may approximate the total contained in the brain (Tamgüney *et al.*, 2009). These data support a fecal-oral route as a likely natural mechanism for the transmission of CWD prions among deer and other susceptible cervid species and possibly for scrapie prions among sheep and goats. Prion shedding through much of the disease course would facilitate exposure of conspecifics and susceptible sympatric species, as well as geographical spread as cervids move between seasonal ranges (Tamgüney *et al.*, 2009).

The potential for CWD transmission by exposure to contaminated soil is also possible since cervids are known to ingest and inhale large amounts of soil, up to 30g/day (Beyer *et al.*, 1994). Brown and Gajdusek (1991) originally demonstrated detection of prion activity after exposure to soil, where scrapie infected hamster brain homogenate mixed with soil and stored outdoors in buried flowerpots remained infectious three years later. Johnson *et al.* (2006) have shown that prions bound to soil minerals remain infectious and that prions bound to soil

are more infectious than unbound prions (Johnson *et al.*, 2007). Uptake of the prion protein upon ingestion or inhalation could also be enhanced by binding to soil, either by increasing residence time in the animal or improving the prion's accessibility to uptake mechanisms (Saunders *et al.*, 2008).

1.1.4.3.7 Human susceptibility

1.1.4.3.7.1 Epidemiological studies

In 2001 the University of Colorado School of Medicine, in conjunction with the Colorado Department of Public Health and Environment Human Prion Disease surveillance program, established a protocol for conducting thorough clinical evaluation, neuropathological assessment and prion protein testing to assess the possibility of CWD transmission to humans. The main purpose of the surveillance program was to identify patients with an atypical neurological or neuropsychiatric presentation, unusual epidemiological features for prion disease such as young age or significant exposure to potentially CWD infected deer or elk. Currently, the program involves urgent review of potential cases of human prion disease, screening 5 to 10 cases each year since its inception. Under the program, any human prion disease encountered is a physician-reportable condition, requiring an investigation to be conducted, autopsies performed on all suspected prion disease cases and epidemiological studies to be initiated. These combination approaches have revealed little evidence for potential transmission of CWD to humans. However, there were two examples in which the possibility of human transmission was considered high, based on the clinical presentation and exposure history (Anderson *et al.*, 2007). The first case involved a 52-year-old

woman with a one year history of progressive memory loss, language impairment, visuospatial disturbance, and myoclonus (spasmodic involuntary twitching of a muscle or a group of muscles). The case patient had been a histology technician in a laboratory that processed tissue specimens from deer and elk with CWD and had handled specimens without wearing gloves for years. Both she and her family expressed significant concerns about the possibility of transdermal or aerosol transmission of CWD. Her family history was negative for dementia and other neurological disorders. Brain magnetic resonance imaging showed mild diffuse volume loss, and electroencephalopathy demonstrated mild diffuse slowing. Other laboratory studies were unremarkable except for a weakly immunostaining 14-3-3 tau protein band, which is an indeterminate finding for the diagnosis of prion disease. Genetic testing of the prion protein gene revealed methionine homozygosity at codon 129, similar to 100% of the clinical cases of the human prion disease variant Creutzfeldt-Jakob disease (vCJD) found to date, yet only representative of 40% of the general population. Post-mortem brain biopsy results were negative for the presence of protease resistant prion protein but showed definite Alzheimer disease with numerous neuritic plaques and tau-positive neurofibrillary tangles. Further analysis of the brain tissue at the National Prion Disease Pathology Surveillance Center at Case Western Reserve University in Ohio, was negative for prion disease by Western blot analysis (Anderson *et al.*, 2007).

The second case involved a 25-year-old man with a 4-month history of progressive gait disturbance, myoclonus, hallucinations, slowed cognition, impaired attention and memory loss (Anderson *et al.*, 2007). He had hunted deer

and elk for years in southern Wyoming, an area considered endemic for CWD, and had cooked and eaten the field dressed meat on a regular basis. His family history was significant in that his mother had died of a dementing disease at 40 years of age, although there was neither a clinical diagnosis nor an autopsy. Brain magnetic resonance imaging findings were unremarkable, and electroencephalopathy demonstrated 1-Hz high-amplitude periodic sharp wave complexes considered normal. Other laboratory studies had negative results. Testing for the 14-3-3 tau protein had positive results, but the cerebrospinal fluid condition was normal. A detailed autopsy examination and referral of the brain to the National Prion Disease Pathology Surveillance Center showed the presence of protease resistant prion protein by Western blot analysis indicating a prion disease. Genetic evaluation revealed the proline-to-leucine mutation at codon 102 of the human prion protein gene confirming the diagnosis of Gerstmann-Sträussler-Scheinker syndrome, a familial genetic prion disease (Anderson *et al.*, 2007).

Despite reported exposure to potentially CWD-positive tissues, alternate diagnoses were confirmed by clinical, neuropathological and genetic evaluation for both patients. In both cases, patients were found to have rare neurological diagnoses: early onset Alzheimer disease in the first case and a familial form of prion disease, proven by the identification of an established gene mutation, for the second case. These examples underscore the importance of a thorough clinical, neuropathological and genetic evaluation before a neurological or neuropsychiatric disorder in persons exposed to potentially infected cervids can be attributed to CWD. Even though CWD has been endemic in areas of Colorado

and Wyoming for decades, the incidence of Creutzfeldt-Jakob disease (CJD) and the age distribution of CJD patients in these two states are similar to those seen in other parts of the United States (Anderson *et al.,* 2007).

1.1.4.3.7.2 Laboratory studies

Laboratory methods can also be used to assess the possible transmission of infectious prions between species. With BSE and vCJD, several lab studies provided crucial evidence that helped establish a causal link between the two diseases by characterizing the molecular similarities of the agents and determining the lesion profile and incubation period patterns (Bruce *et al.,* 1997; Scott *et al.,* 1999; Collinge *et al.,* 1996). It was determined that the likelihood of successful interspecies transmission is influenced by the degree of homology of the infecting prion protein with that of the host endogenous prion protein and gave rise to the concept of a species barrier; a natural barrier to transmission which needs to be overcome before an infecting prion strain can cause disease in a recipient host (Belay *et al.,* 2004). *In vitro* cell-free conversion reaction experiments were developed to assess the degree of molecular compatibility of disease-associated prions from one species with normal prion protein obtained from another species (Raymond *et al.,* 2000; Raymonds *et al.,* 1997). Such experiments specifically addressed the likelihood that an infecting prion would potentially initiate the formation and propagation of pathogenic prions if it came into contact with normal prion protein. A cell-free conversion experiment indicated that CWD-associated prions can convert human prion protein into its abnormal conformer, but at a very low rate (Raymond *et al.,* 2000). The efficiency of this conversion

was more than 14-fold weaker than the homologous conversion of cervid prion protein and more than 5-fold weaker than the homologous conversion induced by CJD-associated prions; however, a low level of compatibility between infecting prions and host prion protein does not necessarily rule out natural interspecies transmission. Several other factors may determine the *in vivo* transmission efficiency including dose, strain of the agent, route of infection, stability of the agent inside and outside the host, and the efficiency of agent delivery to the nervous system (Belay *et al.*, 2004).

A recent study using protein misfolding cyclic amplification (PMCA), a highly sensitive method for *in vitro* amplification conversion of normal prion protein into the abnormal conformer, demonstrated that cervid PrP^{Sc} can induce the conversion of normal human prions albeit with the restriction that CWD prions had to be stabilized and adapted either by PMCA or *in vivo* passage through cervidized transgenic mice (Barria *et al.*, 2011). The newly generated human PrP^{Sc} exhibited a distinct biochemical pattern that differed from that of any of the currently known forms of human PrP^{Sc}. This could suggest that the transmission barrier may be a dynamic process that depends on the strain and moreover the degree of adaptation of the strain. If these findings are corroborated by infectivity assays, they may imply that certain CWD prion strains could be infectious humans and that the likelihood progressively increases with CWD spreading. Of note, when field isolates of CWD brains were used as a template, no conversion of human PrP^{C} was achieved, which may question the relevance of the *in vitro* data (Gilch *et al.*, 2011).

Studies using transgenic mouse lines that over-express the human prion protein have been performed to determine human susceptibility to CWD. These transgenic mice have been utilized in extensive transmission studies of human and animal prion disease with some lines susceptible to BSE and vCJD prions, allowing for comparison with CWD. To date, four studies have reported that transgenic mice expressing human prion protein are resistant to CWD prions (Kong *et al.*, 2005; Tamgüney *et al.*, 2006; Sandberg *et al.*, 2010; Wilson *et al.*, 2012). The first of these studies used two lines of transgenic mice expressing human prion at either one or two times the endogenous level of mouse brain. After inoculation with CWD-infected elk brain homogenate none of these transgenic mice showed clinical signs of prion disease or detectable accumulation of abnormal PrP^{Sc} by either immunohistochemistry or immunoblotting (Kong *et al.*, 2005). The second study used hemizygous transgenic mice expressing human PrP at two times the endogenous level of murine PrP expression in mouse brain (Tamgüney *et al.*, 2006). No evidence of clinical prion disease was observed following intracerebral inoculation with CWD infected elk, mule deer or white tailed deer brain homogenates; however, subclinical infection was not excluded. The third study looked at transmitting mule deer CWD prions to lines of transgenic mice overexpressing human PrP two to six-fold with either methionine or valine at polymorphic residue 129 (Sandberg *et al.*, 2010). These mice are known to be susceptible to a wide range of human prion diseases (Wadsworth *et al.*, 2008) as well as BSE (Asante *et al.*, 2002). The mice were resistant to CWD infection arguing that the transmission barrier associated with this prion strain/host combination was greater than that observed with classical BSE prions

(Sandberg *et al.*, 2010). However it is still possible that CWD may be caused by multiple prion strains so further studies of this type will be required to evaluate the transmission properties of distinct prion strains as they are characterized (Sandberg *et al.*, 2010). The fourth study involved inoculating a CWD isolate into gene-targeted transgenic (Tg) mice expressing the human prion protein (PrP) (Wilson *et al.*, 2012). Upon challenge, gene-targeted Tg mice expressing human PrP did not show any signs of disease pathology suggesting the presence of a substantial transmission barrier between CWD and humans (Wilson *et al.*, 2012).

The best possible models to study transmission of animal prion diseases to humans are probably the non-human primates. Inoculation of BSE prions into *Cynomolgus* macaques provided the first hints that BSE might be transmissible to humans (Lasmezas *et al.*, 1996). To assess the susceptibility of non-human primates to CWD, Marsh *et al.* (2005) intracerebrally inoculated two squirrel monkeys with brain tissue from a CWD infected mule deer. The CWD inoculated squirrel monkeys developed a progressive neurodegenerative disease and were euthanized at 31 and 34 months post infection. Brain tissue from the CWD infected squirrel monkeys contained the abnormal isoform of the prion protein and displayed spongiform degeneration. This was the first reported transmission of CWD to primates. This evidence that at least one species of non-human primate was susceptible to CWD weakened any conclusions that humans may be protected from CWD by a species barrier (Marsh *et al.*, 2005). Several years later, Race *et al.* (2009) expanded on the work of Marsh *et al.* (2005) by using two non-human primate species, squirrel monkeys and cynomolgus macaques, as human models for CWD susceptibility. CWD from 8 different pools representing wild

and captive cervids, including mule deer, white tailed deer and elk, from separate regions in the United States, were inoculated into these two species by intracerebral or oral routes. After intracerebral inoculation of squirrel monkeys, seven of eight CWD isolates induced a clinical wasting syndrome within 33-53 months. The monkeys' brains showed spongiform encephalopathy and protease resistant prion protein (PrP^{Res}) diagnostic of prion disease. Oral transmission of TSE is generally 1000-fold less effective than direct intracerebral challenge and results in longer incubation periods and lower efficiency of disease transmission (B. Race *et al.*, 2009). After oral exposure, two of 15 squirrel monkeys had PrP^{Res} in brain, spleen, and lymph nodes at 69 months post infection. In contrast, the cynomolgus macaques did not show any evidence of clinical disease suggesting that these two species of nonhuman primate differed in susceptibility to CWD, although results in cynomolgus macaques might be of higher relevance since they are evolutionarily closer relatives of humans than squirrel monkeys (B. Race *et al.*, 2009).

In another recent study, Béringue *et al.* (2012) showed that in transgenic mice expressing human or ovine prion protein, lymphoid tissue is more susceptible to cross-species transmission than neural brain tissue, which may prompt a reevaluation of the effectiveness of species barriers. In many prion infections, including natural sheep scrapie and vCJD, as well as experimental rodent models, prions propagate in the lymphoreticular system (LRS: spleen, lymph nodes, tonsil, appendix, and other gut-associated lymphoid tissues), and this may precede (and in some cases be necessary for) neuroinvasion (Wadsworth *et al.*, 2001). This study found that no clinical disease resulted when they

challenged mice expressing ovine PrP^{C} with CWD prions; however, almost all of the mice had evidence of LRS prion infection at death. Only a small minority of mice, which lived more than 500 days, had detectable brain PrP^{Sc}. The transmission barrier was more readily crossed in the LRS than in the brain. A similar situation was found in mice expressing human PrP^{C} and challenged with BSE prions where little evidence of clinical disease was noted and few subjects had brain infections yet spleen infections were common. Even though humanized mice were not challenged with CWD prions, the data still call for a closer examination of extraneural PrP^{Res} in humanized or primate models when assessing any zoonotic potential of animal prions. Prion detection in the lymphoid tissue whenever possible might also be considered for the surveillance of people exposed to CWD-infected tissue. Important data relating to the transmissibility of CWD to humans may otherwise be missed (Béringue *et al.*, 2012).

To date, no solid evidence of human susceptibility to chronic wasting disease has been found. Most epidemiological studies do not suggest transmission of CWD in humans. Despite *in vitro* studies indicating inefficient conversion of human prion protein by CWD associated prions, no human cases of prion disease with strong evidence of a link with CWD have been identified. The absence of any increase in CJD-like disease incidence in Colorado and Wyoming suggests that the risk of transmission of CWD to humans is low. Laboratory studies involving animal models have also indicated that a strong species barrier exists; however, the low level transmission of BSE to humans and the resulting vCJD indicate that with enough exposure the species barrier may not completely protect humans from animal prion diseases.

1.1.4.3.8 Public health: surveillance and prevention

Common approaches for CWD surveillance, in order to determine CWD distribution, include collection and testing of deer, elk or moose that show clinical signs of CWD; sampling of hunter-harvested cervids during annual hunting seasons for CWD examination; sampling of vehicle killed cervids and depopulation of infected game farms upon detection. In addition to surveillance, preventative measures intended to reduce opportunities for further spreading and establishment of new foci include regulations preventing the translocation or movements in commerce of live animals and carcass parts from endemic regions or directly neighboring wild populations to other areas; regulations prohibiting the release of animals from captive herds into free-ranging populations; recommendations to double fence established game farms in CWD-endemic areas and discouraging the establishment of new captive facilities in such areas (Miller *et al.*, 2000).

1.2 Diagnostic detection

There are many challenges associated with the development of a detection method capable of diagnosing a prion disease. Similarities in the amino acid sequence of PrP^{Sc} and PrP^{C} make distinguishing the infectious from the normal prion protein difficult. The extensive tissue distribution associated with some but not all prion diseases complicates finding representative samples. The high levels of sensitivity required to detect the low levels of PrP^{Sc} found in the tissues as well

as the necessity to correlate detection with animal bioassay infectivity ultimately limit the options available for an accepted diagnostic test.

PrP^{Sc} is the only known disease-associated marker of a prion disease so most approaches have focused on detection of PrP^{Sc} in host tissues. Knockout mice that lack the prion gene *prnp* (*prnp*$^{0/0}$) are completely resistant to prion disease (Bueler *et al.*, 1993) and PrP^{Sc} has been shown to co-purify with infectivity (Gabizon *et al.*, 1988). Due to the similarities between the normal and infectious prion protein, methods of detection have had to rely on the unique biophysical properties of PrP^{Sc}. Strategies have focused on different approaches in order to differentiate the two isoforms: conformational differences (Baldwin *et al.*, 1994), resistance to proteases (McKinley *et al.*, 1983), treatment with heat and/or acid, insolubility in chaotropic agents like urea or guanidinium chloride (Dabaghian *et al.*, 2006). Approaches are typically followed by antibody detection.

Post-mortem confirmatory tests utilize heavily infected areas of the brain such as the obex for detection of PrP^{Sc}. Ante-mortem diagnostic tests have to focus on more readily accessible samples with PrP^{Sc} deposition. These tests rely on intact tissues typically known to be infected with prions during the course of the disease. Unfortunately, the varying distribution and extent of the PrP^{Sc} in the tissue selected, the unknown stage of the disease and the diverse fluid distribution of PrP^{Sc}, all of which is dependent on the specific prion disease and species infected, limits their applicability for ante-mortem testing. In addition, most diagnostic tests are time consuming and technically demanding, requiring sedation of the animal, and because the tissues cannot be repeatedly sampled, the

applicability for pre-clinical diagnosis or confirmation at various time points is limited.

1.2.1 Immunohistochemistry

Immunohistochemistry (IHC) became the gold standard for TSE diagnosis in the 1980s and is still used today to detect the characteristic appearance of spongiform encephalopathy in post-mortem brain tissue. Similar pathologies associated with non-infectious spongiform-like encephalopathies had originally been problematic for pathologists, but following the discovery of scrapie-associated fibril as primarily being composed of prion protein (Merz *et al.*, 1981), antibody based approaches could be utilized. Current methods of IHC use formic acid and high temperature (autoclaving) to destroy the PrP^{C} in the tissue prior to immunostaining in order to distinguish between the PrP^{C} and PrP^{Sc}. The IHC method can be used to detect PrP^{Sc} in the 3rd eyelid of sheep (O'Rourke *et al.*, 2000), rectal lymphoid follicles and retropharyngeal lymph nodes in deer (Wolfe *et al.*, 2007), the rectal mucosa of sheep (Espenes *et al.*, 2006; González *et al.*, 2006) and the palatine tonsil of sheep (Van Keulen *et al.*, 1996; Schleuder *et al.*, 1998) and deer (Wild *et al.*, 2002). Many factors can affect detection ability including the quality of the sample sections as well as the sample preparation and the specificity of the antibody to the species being evaluated.

1.2.2 Western blot

Currently, the Western blot (WB) assay is the most validated and characterized of the screening protocols, allowing for a time frame of 1-2 days for the measurement of PrP^{Sc} (Ingrosso *et al.*, 2002). Coupled with sodium, dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and antibody detection, the WB assay for PrP^{Sc} detection uses a combination of protease digestion and heat degradation. Protease digestion with Proteinase K (PK) is used to distinguish between the protease-resistant form, PrP^{Sc}, and the protease susceptible form, PrP^{C}. The PK treatment is used to completely hydrolyze the PrP^{C} but only to cleave off the amino-terminus end of the PrP^{Sc}. This results in a protease resistant core protein remaining after digestion that is 27-30kD in size or approximately 142 amino acids in length. After protein separation by gel electrophoresis and antibody detection (e.g., chemiluminescence), the PrP^{Sc} can be visualized on the blot as three bands varying in molecular mass depending on their glycosylation status (di-, mono-, or unglycosylated). The intensity of the distinct banding pattern can be quantified to determine the amount of infectious material present in the original sample. Using 263K scrapie infected hamster brain homogenates, the detection limit has been shown to be around 10-20 pM or 5-10 pg of PrP^{Sc} (Lee *et al.*, 2000). Depending on subtle band shifts in molecular weight and comparative band intensity, the banding pattern may also be used to differentiate between different strains of prion disease such as typical versus atypical scrapie in sheep (Gavier-Widén *et al.*, 2004).

The advantages of the WB include the quantification of protease resistant PrP^{Sc} directly from a sample and the ability to distinguish between different prion

strains and thus different prion diseases within a single species (scrapie versus BSE in sheep) (SH113). The disadvantages include reduced sensitivity due to the destruction of potentially protease sensitive PrP^{Sc} as well as the time consuming and labor intensive requirements inherent in the assay (Safar *et al.*, 2005).

1.2.3 ELISA

Enzyme-linked immunosorbant assay (ELISA) platforms have also been developed for the diagnostic detection of PrP^{Sc}. Like the WB assay, most ELISA based platforms require the protease digestion of PrP^{Sc} in order to distinguish it from PrP^{C}. Digestion is followed by antigen capture of the PrP^{Sc} through immobilization of primary antibodies into a plastic well with subsequent secondary antibody detection through chemiluminescence. The ELISA-based platforms are currently used as a screening tool, requiring confirmation by WB, but the improved detection limit (i.e., 2 pM) and high volume processing capacity with its reduced time and labor components make it a valuable upstream tool (Ingrosso *et al.*, 2002).

1.2.4 Protein Misfolding Cyclic Amplification

Protein misfolding cyclic amplification (PMCA) was originally developed by Saborio *et al.*, (Saborio *et al.*, 2001) to facilitate preclinical detection of prions in peripheral tissues, most notably blood. Conceptually analogous to polymerase chain reaction's ability to amplify DNA, PMCA amplifies PrP^{Sc} present in a sample to detectable levels for conventional detection methods such as the WB.

This approach mimics the pathological process *in vitro* by incubating PrP^{Sc} in the presence of excess PrP^{C} to allow expansion of aggregates of PrP^{Sc} that are then dispersed by sonication pulses to generate smaller units in order to promote the formation of new aggregates. The quantity of PrP^{Sc} formed is directly dependent on the number of expansion/sonication cycles performed (Grassi *et al.*, 2008). PMCA has contributed greatly to our knowledge related to the protein hypothesis (Prusiner, 1998; Soto, 2011), species barrier (Castilla *et al.*, 2008), prion strains (Castilla *et al.*, 2008) and has been used to assess the occurrence of prions in various body tissues (Soto *et al.*, 2005), fluids (Castilla *et al.*, 2005; Gonzalez-Romero *et al.*, 2008), environmental samples (Nagaoka *et al.*, 2010), water (Nichols *et al.*, 2009), and from animals and areas where infectivity is thought to occur, and at a level of sensitivity never before seen (Saá *et al.*, 2006).

Originally, the PMCA method involved mixing prion-infected brain homogenate (IBH) diluted in a >1000 fold excess of normal uninfected brain homogenate (NBH) with each PMCA cycle allowing amplification of PrP^{Sc} during a 1 hour incubation at 37°C and subsequent disruption of the PrP^{Sc} aggregates by five 1 second sonication bursts. The constant temperature was used to mimic the temperature found in most warm-blooded mammals (Lucassen *et al.*, 2003). Incubation facilitated conversion and aggregation, while sonication multiplied the number of small aggregates available to induce PrP^{Sc} conversion. After 5 amplification/incubation cycles the newly formed PrP^{Sc} constituted greater than 95% of the total PrP^{Sc} present (Saborio *et al.*, 2001). In the earliest studies, amplification was modest (10- to 50-fold), but optimization and automation

subsequently enabled amplifications of several million-fold to be achieved (Grassi *et al.*, 2008).

A major change in the method was the incorporation of a programmable sonicator and a 96-well plate format, which enabled high through-put assays and eliminated the potential for cross-contamination between samples (Castilla *et al.*, 2005). Each round consisted of 20 cycles with a 40 second sonication pulse every 30 minutes. Upon completion of each round, a small aliquot of the amplified samples were taken and diluted 10-1000-fold into fresh NBH to carry out the subsequent rounds of PMCA. This serial PMCA (sPMCA) was shown to be continued successfully even after the original seeds were diluted up to 10^{55} –fold, suggesting that PrP^{Sc} could be replicated infinitely *in vitro* as long as fresh NBH was supplied. Furthermore and most importantly, the products of the sPMCA retained the characteristics of the original PrP^{Sc} seed such as infectivity, glycosylation pattern, amino acid composition, PK resistance, Fourier transform infrared spectroscopy profile, electron microscopy profile, heat-resistance profile and resistance to denaturation by chaotropic agents like urea or guanidinium chloride (Castilla *et al.*, 2006).

The most significant advantage of the sPMCA assay is its level of sensitivity, able to amplify as little as ~26 molecules of protein monomers (*i.e.* $1x10^{-12}$ dilution) of scrapie hamster brain to detectable levels by WB after seven rounds (Saá *et al.*, 2006) and unlike previous *in vitro* conversion methods, the PrP^{Sc} generated by PMCA was found to be infectious in animals and sufficient for disease (Castilla *et al.*, 2005). Since the original PrP^{Sc} seeds were diluted beyond the minimum infectious dose, it would appear that infectivity was due to the

newly synthesized PrP^{Sc} (Castilla *et al.*, 2005). The disease characteristics were also conserved in the PMCA product and resulted in identical clinical signs and vacuolation patterns as the original seed templates. In addition, the pathogenicity of the *in vitro* generated PrP^{Sc} appeared to be stable upon serial transmission (Castilla *et al.*, 2005). One difference, though, suggests that PMCA may be less efficient at generating infectious prion particles than *in vivo* systems: the *in vitro* generated PrP^{Sc} product exhibits longer incubation periods in animals than an equal amount of brain-derived PrP^{Sc}. It was thought that transmission of disease may be hindered by the sonication process itself where damage at a molecular level may occur or the presence of detergents, added during the brain homogenate preparation, might denature cellular protein factors or disrupt the native mechanism for the *in vivo* conversion of PrP^{C} to PrP^{Sc} (Ryou & Mays, 2008).

To address this concern, studies using a non-denaturing protocol were developed by omitting the use of sonication and anionic detergent sodium dodecyl sulfate in order to more closely mimic an *in vivo* system (Lucassen *et al.*, 2003; Deleault *et al.*, 2005). Instead of sonication, continuous shaking was used. This modified version of PMCA utilized digested PrP27-30 (PrP-res) as seeds to convert mature, mammalian PrP^{C} partially purified from normal brain homogenate by detergent solublization along with immunopurification. Unfortunately these conditions led to significantly less amplification (~2-fold) (Lucassen *et al.*, 2003). Supplementation of polyanionic compounds such as synthetic poly A^{+} RNA in the reaction increased PrP-res formation to ~10-fold (Deleault *et al.*, 2005). More PrP-res formation was achieved if sonication was applied but no periodic sonication resulted in failure of PrP-res formation, suggesting that sonication

should be an essential part of the revised method (Deleault *et al.*, 2007). Incubation of PrP27-30 and PrP^{C} highly purified by a combination of several chromatographic steps along with synthetic poly A^{+} RNA molecules resulted in efficient PrP-res formation, even in the absence of PrP27-30 seeds implicating *de novo* generation of PrP^{Sc} (Deleault *et al.*, 2007). Similar to seeded PMCA products, *de novo* generated PrP^{Sc} was infectious when inoculated into animals and exhibited almost equivalent infectivity, neuropathological characteristics and clinical symptoms to naturally acquired prions found in a diseased brain (Deleault *et al.*, 2007).

1.3 Research Rationale, Hypothesis and Objectives

1.3.1 Research Rationale

Collectively, the data suggest that limiting environmental burden and shedding of CWD in infected animals may be a critical component in minimizing contagion within herds and disseminating the disease geographically. However, critical knowledge gaps currently exist regarding the contagious nature of CWD: i) when does an animal become contagious during the course of infection; ii) what tissues and/or bodily fluids and excrements act as vehicles for PrP^{CWD} secretion or shedding; iii) at what point during the course of the disease does secretion or shedding from these tissues, secreta and excreta occur; iv) what is the burden of shedding in these tissues, excreta and secreta; v) how well do traditional diagnostic methods (ELISA, IHC and WB) correlate with onset of contagion (i.e. how soon pre- or post PrP^{CWD} lymphoid positivity does an animal begin shedding

infectious prions into the environment); and vi) do animals become infected through direct animal to animal contact and/or indirectly through the environments where prions can accumulate to doses necessary to establish infection.

The development of a robust, sensitive and cost effective diagnostic test for early detection of CWD infection in bodily tissues, excreta/secreta and environmental samples such as soil would substantially enhance the ability to monitor, detect and eliminate CWD in captive herds and control CWD spread in natural populations. Moreover, these tools would fill in knowledge gaps related to understanding contagion in CWD-infected cervids. These tools would dramatically enhance the ability of wildlife management agencies to conduct CWD surveillance efforts, monitor the effect of management actions on CWD distribution, and develop high resolution statistically robust predictive models of disease transmission, with the ultimate goal of reducing the potential health risks to animals and consequently humans who consume CWD-infected cervids as a food source.

1.3.2 Hypothesis

Protein misfolding cyclic amplification (PMCA) is an ultrasensitive method for detection of chronic wasting disease (CWD) in cervids, capable of detecting prions in tissues and fluids of infected cervids at levels equal to or greater in sensitivity than existing diagnostic methods. The application of this method will

be important in characterizing patterns of contagion and transmission of PrP^{CWD} in naturally infected cervids.

1.3.3 Objectives

1.3.3.1 Objective 1: Adapt, optimize and standardize a PMCA PrP^{CWD} protocol for detection of PrP^{CWD} in cervids.

Previous work carried out in our laboratory focused on the development and application of PMCA for detection of 263K scrapie in hamsters and relatively little work across the prion research field had been done to adapt PMCA to detection of CWD up until the onset of this thesis research (2008). The focus of this objective was to optimize conditions of PMCA for amplification of PrP^{CWD} from infected animals and include evaluative parameters such as sonication power, duration of incubation, number of cycles and rounds of PMCA, buffer conditions, and temperature. In addition, Western blot detection was also optimized and included PK sensitivity analysis and general signal detection methods (i.e., chemiluminescence). The optimized conditions were then tested across a number of CWD isolates and across a number of cervid species including white-tailed deer, mule deer, and elk (Chapter 3).

1.3.3.2 Objective 2: Validation of the PMCA PrP^{CWD} protocol in experimentally and naturally-infected cervids.

Traditional diagnostic methods incorporate the use of various tissues in confirmation of CWD testing (obex, retropharyngeal lymph node, tonsil) using multiple test platforms, including enzyme-linked immunosorbent assays (ELISA),

immunohistochemistry (IHC) and Western blot (WB). A comparative assessment was performed to examine the correlation of the optimized PMCA PrP^{CWD} protocol to traditional diagnostic methods for detection of PrP^{CWD} in experimentally and naturally infected (farmed) white-tailed deer (WTD) as well as wild cervids (WTD and mule deer) in endemic areas of disease. As with all clinical tests, diagnosis of disease is a function of the sensitivity/specificity of the diagnostic test. Therefore, it is conceivable that application of ultrasensitive tests such as PMCA for detection of CWD in naturally occurring animals may reveal that prevalence rates in nature could be higher than previously thought. The research related to this objective is presented in Chapter 4 of this thesis.

1.3.3.3 Objective 3: Assess distribution of CWD in tissues and fluids (i.e., secreta and excreta) of infected cervids.

A critical component to this phase of the research was to examine the time-dependent tissue distribution of CWD prions in infected animals using PMCA as an ultrasensitive test. This phase of the research has two important implications for the field. Firstly, the data are critical for better understanding a time-dependent accumulation of prions in specific WTD tissues, particularly those tissues that are routinely consumed by humans (i.e., spleen, kidney, liver, skeletal muscle, heart, and adrenal glands). Recent data suggest that prions may accumulate to high levels in certain tissues well in advance of the clinical onset of disease (Hill and Collinge 2003). Consumption of these tissues may lead to an increased risk of exposure (i.e., dose) in human populations where CWD infections are endemic. Secondly the data are important for determining whether

prions can be shed in the secreta (saliva) or excreta (feces and urine) of infected animals, and consequently when shedding actually occurs during the course of infection. The data are important in understanding when an animal potentially becomes contagious during the course of infection. The results pertaining to this objective are presented in Chapter 5.

The most significant findings, importance of these research findings and future areas of research are subsequently presented in Chapter 6.

CHAPTER 2: MATERIALS AND METHODS

2.1 Introduction

Previous work carried out in our laboratory focused on the development and application of PMCA for detection of 263K scrapie in hamsters (Braithwaite, 2010). A PMCA method was developed that was capable to amplifying and detecting PrP^{Sc} down to a one in one trillionth dilution (10^{-12}) of an infected hamster brain in just 2x16 hour cycles (40 seconds of sonication followed by a half hour of incubation at 37°C). These parameters were be used as a starting point for amplification and detection of CWD by PMCA.

2.2 Conversion buffer solutions and preparation

2.2.1 Conversion buffer reagents

Phosphate buffer saline (PBS) was prepared as a 0.66X PBS, pH 7.4 by adding 150mL double distilled water to 1 PBS tablet (BioBasic Inc., Toronto, ON, catalogue #PD0435). A 5M NaCl solution was prepared for use in the conversion buffer (CB) and was discarded six months after the preparation date (Fluka/Sigma-Aldrich, Toronto, ON, catalogue #71376). Triton X-100 (Calbiochem, San Diego, CA, catalogue #648466) was diluted to a stock solution with a final concentration of 20% in 1X PBS in order to overcome pipetting difficulties associated with the solution's viscosity. The 20% Triton X solution was prepared at least one day in advance of the CB preparation to allow the viscous detergent to completely mix with the PBS. A 0.5M solution of

ethylenediaminetetraacetic acid (EDTA) was purchased from Gibco (Invitrogen Canada Inc., Burlington, ON, catalogue #15575-038) and added directly to the CB preparation. A complete Protease Inhibitor Cocktail was purchased from Roche Diagnostics (Laval, QC, catalogue #1836145) and added according to the manufacturer's instruction of 1 tablet to a 50mL volume of 1X PBS.

2.2.2 Conversion buffer preparation

The CB was prepared according to Castilla *et al.,* 2006 with the addition of 5mM of EDTA as described by Castilla *et al.*, 2004. Solutions and components were added to 0.66X PBS in the order and concentration listed in Table 2.1 and mixed well to ensure the Complete Protease Inhibitor tablet was completely dissolved. The CB was prepared at least 2 hours in advance of use.

Table 2.1: Conversion buffer preparation.

Component	Final Concentration	Volume (50mL)
5M NaCl	0.15M	1.5mL
0.5M EDTA	5mM	0.4mL
20% Triton X-100	1%	2.5mL
Complete Protease Inhibitor Cocktail	1x	1 tablet

2.3 Proteinase K preparation

Proteinase K powder (Promega, Madison, WI, catalogue #V3021) was reconstituted in ddH_2O to 20mg/mL stock solution and aliquoted in 100µL volumes before freezing to -80°C. This PK stock solution was diluted to

250µg/mL with PK digest buffer (4% SDS [BioRad, Hercules, CA, USA, catalogue #161-0301], 0.1% Triton X-100, in 0.66x PBS) just prior to use.

2.4 Animal handling protocols

All animal handling and tissue preparation protocols used in this thesis adhered to the Canadian Council of Animal Care (CCAC-Canada) guidelines. Protocols were approved through onsite Animal Care Committees at either the Canadian Food Inspection Agency – Ottawa Laboratory Fallowfield (CFIA-OLF) or the University of Alberta.

2.5 Tissue and sample collection

2.5.1 PMCA PrP^{Sc} normal hamster brains

For the scrapie PMCA assay, whose conditions were used as a starting point for developing the CWD PMCA assay, normal hamster brains were prepared from four to six week old female Syrian Golden hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA). Hamsters were euthanized shortly after delivery by exposure to excess carbon dioxide (dry ice in a kill box). Reflexes using a toe pinch and breathing were measured to confirm hamsters had expired prior to further processing. Upon confirmed death, each hamster was pinned by its extremities to a polystyrene (Styrofoam) board. Scissors were used to cut the skin of the abdomen laterally across the body of the hamster and then centrally up to the heart ensuring not to disturb/puncture any organs. Excess skin was pinned back and a needle (20 gauge) connected to a peristaltic pump was

immediately inserted in the left ventricle. Cold PBS with 5 mM EDTA was perfused throughout the hamster circulatory system using the pump. After the 25mL of PBS/EDTA solution had been flushed through the hamster's circulatory system at a rate of 5mL/minute, the needle and pins were removed. The hamster was then flipped over, with dorsal side up, and scissors were used to cut and sever the spinal cord. Additional cuts were made on either side of the head from the severed spinal cord towards each ear. A lateral cut was then made between the eyes to detach the front of the skull from the brain. One final cut through the center of the skull bone on the top of the brain was made so that the sides of the skull could be easily removed to expose the entire brain. A well-perfused brain was completely white, lacking any pink or red staining associated with hemorrhage. The brain was then gently removed from the cranium. Each hemisphere, along with as much of the obex as possible, was placed in a small snap cap tube (1.5 mL Eppendorf, Mississauga, ON, catalogue # 2236 334-4). Tubes containing brain tissue were collected on dry ice and then stored at -80°C until required.

2.5.2 PMCA PrP^{Sc} infected hamster brains

Three to four week old female Syrian Golden hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA) were exposed orally (100 μL) or by intraperitoneal (i.p.) injection (50 μL) to an inoculum of scrapie positive brain homogenate upon receipt at the CFIA-OLF Animal Facility. The positive scrapie 263K inoculum consisted of a 10% pooled brain homogenate in

PBS with ampicillin. Both ELISA and IHC confirmed all infected animals positive. The infectious dose of brain homogenates was subsequently determined to be $10^{9.94}$ ID_{50} per gram of brain tissue as confirmed by hamster infectivity endpoint titration assays (data supplied by Dr. Balachandran, CFIA).

Syrian Golden hamsters displaying clinical signs of scrapie, typically 95-110 days post inoculation (dpi), were euthanized with carbon dioxide and the brains harvested as quickly as possible. Perfusion was not performed on infected hamster brains. Half the brain was preserved in formalin and the other half frozen in an inventory repository (-80°C). A macroscopic necropsy of the abdominal cavity was completed to rule out any other disease process. For the purposes of PMCA only frozen inventory was utilized.

2.5.3 PMCA PrP^{CWD} normal TgElk brains

Recent data published during the course of this thesis suggested that the use of normal cervid brain homogenate as a source of prion protein for PMCA represented an inefficient template for amplification in PMCA (Kurt *et al.*, 2007). The limited number of studies attempting to adapt PMCA to the detection of CWD in cervids employed the use of transgenic cervidized mice as a source of normal prion protein. For this reason, the transgenic TgElk mouse line (LaFauci *et al.*, 2006) was used as a source of normal prions for development of the PMCA assay for CWD. The suitability of the TgElk mouse line for applications related to PMCA was not yet explored prior to the commencement of this thesis. Infectious PrP^{CWD} was provided as a brain homogenate sample from experimentally infected

white-tailed deer (Dr. Aru Balachandran, Canadian Food Inspection Agency [CFIA], Ottawa, Ontario) or elk (Dr. Catherine Graham, Canadian Food Inspection Agency, Lethbridge, Alberta).

For the CWD PMCA assay, brains of 3-6 week old TgElk mice (Baltimore Research Institute) were perfused with cold PBS containing 5mM EDTA immediately after being euthanized with CO_2. The brains were removed and frozen at -80°C.

2.5.4 PMCA PrP^{CWD} experimentally and naturally infected deer organs, feces, urine and saliva

Female white-tailed deer (WTD) and red deer (RD) sourced from CWD-free captive herds were group housed in isolation barns at the Canadian Food Inspection Agency, Ottawa Laboratory, Fallowfield (Ottawa, Ontario). Deer were orally inoculated (RD: December 2006 and WTD: December 2008) with a 5mL pool of control positive (20% w/v) white tail deer brain homogenate in saline via syringe directed into the oropharynx. This was done twice, one week apart. All experimentally-infected animals were euthanized after showing clinical signs consistent with CWD and post mortems were performed where obex and retropharyngeal lymph nodes from RD and obex, retropharyngeal lymph nodes, spleen, heart, kidney, liver, muscle, salivary gland, adrenal gland and colon tissues from WTD were harvested and frozen at -80°C. The deer were positive for PrP^{CWD} on IHC, ELISA and Western blot assays. Pooled feces from pens were collected from WTD at 5, 10, 16 and 21 months post inoculation (PI), as well as from individual animals at necrospy. WTD urine was collected post mortem and

WTD saliva was collected a day before the animals were euthanized using a 50mL syringe inside the mouth.

Obex and retropharyngeal lymph node samples from naturally infected WTD were collected for the CFIA from outbreak farms around Saskatchewan. Mule deer obex samples were provided by Alberta Agriculture and Rural Development, from a hunter kill harvest near Wainwright Alberta that took place in November of 2011.

2.6 Preparation of homogenates for PMCA

2.6.1 Normal Brain Homogenate (NBH)

Normal brains (hamster or TgElk) were removed from -80°C and allowed to thaw on ice. An appropriate amount, as determined based on the number of PMCA samples to be performed in any given day, was weighed in a small disposable petri dish for the preparation of a 10% NBH. Typically 1 g +/- 0.2 g of normal (non-infected) brain was placed in the Potter homogenizer (VWR International, Mississauga, ON) with 9mL of CB, and manually disrupted with 20 strokes in the homogenizer. The mixture was then transferred (poured off) to a 15 mL centrifuge tube and for the hamster NBH, placed in the refrigerator (4°C) or left on ice for at least 30 min. The TgElk NBH was not left on ice and was centrifuged immediately after homogenization. Both the hamster and TgElk homogenates were centrifuged at 1000 x *g* (Eppendorf 5804R, Eppendorf Canada**,** Mississauga, ON) for 1 minute and the clarified supernatant used for experimentation. The pellet was discarded along with the zone between clarified supernatant and pellet. The low centrifugal speed/force used to clarify the NBH

preparation was important to ensure that essential co-factors associated with the brain and required for conversion in the PMCA reaction were not removed.

2.6.2 Infectious Brain Homogenate (IBH)

Infected brains (hamster or cervid) were supplied by the CFIA. Infected brains were not perfused when sacrificed and therefore were not free of blood. After sacrifice, infected brains were stored at -80°C until needed for homogenate preparation. A 20% IBH in 1X PBS was prepared in disposable 1.5 mL ribolyzer tubes with zirconium beads (BioRad, Hercules, CA, USA) using the FastPrep homogenizer (Qbiogene Inc., Carlsbad, CA, USA) at a setting of 4 (equivalent to 4.0 m/sec), for 20 sec, followed by a 30 min incubation at 4°C, and centrifugation (Eppendorf 5804R, Eppendorf Canada**,** Mississauga, ON) at 1000 x *g* for 1 min. The supernatant was then removed and the pellet discarded ensuring that the zone between supernatant and pellet was not removed or disrupted. The IBH was then aliquoted into smaller volumes, to avoid issues related to repeated freeze/thaw cycles, and stored at -80°C for future use.

2.6.3 Tissue and organ homogenate preparations from experimentally-infected WTD

Infected white tailed deer obex, retropharyngeal lymph nodes, spleen, heart, kidney, liver, muscle, salivary gland, adrenal gland and colon tissues were supplied by the CFIA. After sacrifice, infected organs were stored at -80°C until needed for homogenate preparation. A 20% organ sample homogenate (w/v) in 1X PBS was prepared in disposable 1.5 mL ribolyzer tubes with zirconium beads

(BioRad, Hercules, CA, USA) using the FastPrep homogenizer (Qbiogene Inc., Carlsbad, CA, USA) at a setting of 4 (equivalent to 4.0 m/sec), for 20 sec, and centrifuged (Eppendorf 5804R, Eppendorf Canada**,** Mississauga, ON) at 1000 x *g* for 40 sec. The supernatant was removed and the pellet discarded ensuring that the zone between supernatant and pellet was not removed or disrupted. The homogenate was then aliquoted into smaller volumes, to avoid issues related to repeated freeze/thaw cycles, and stored at -80°C for future use.

2.6.4 Preparation of fecal homogenates from naturally and experimentally-infected cervids

Fecal pellets were weighed out and brought to 10% w/v with conversion buffer. The pellets were allowed to sit in the buffer for 2 hours with intermittent vortexing to produce a slurry. Debris was removed with a 1 min 1000 x g centrifugation.

2.7 PMCA Sonicator: Misonix 4000

A Misonix Sonicator Model 4000 (Misonix Inc Farmingdale, NY, USA) was used for the work described in this thesis. The sonicator consisted of a microplate cup horn and a generator. The cup horn offers indirect sonication and functions as a high intensity water bath. The generator is the power source for the cup horn and is programmable, allowing the user to preset the duration of individual bursts of sonication, incubation time between cycles of sonication, and the number of cycles to be completed (*i.e.,* total sonication time). The microplate holder consists of a piece of plexiglass with appropriately sized and organized

holes for insertion of 0.2 mL PCR tubes. The holder sits on top of the microplate horn edge, above the water level of the reservoir, and allows the bottom of the PCR tubes (*i.e.*, the sample) to be submersed in the water bath at an appropriate (and consistent) distance from the horn. The sonicator microplate cup horn is housed within an acoustic enclosure that is placed inside a bench top incubator. The temperature setting of the bench top incubator was adjusted to achieve an approximate temperature of 37°C in the water reservoir. In this case, an incubator temperature setting of ~33°C, resulted in a temperature of ~37°C or slightly below (1-2°C) of the water in the cup horn. A temperature at, or slightly below, 37°C was preferable since the temperature of the water in the bath increased as more sonication bursts were completed. Typical sonication cycles consisted of 40 sec of sonication followed by 29 min 20 sec of incubation over the course of 18 hours (37 cycles). Each PMCA round was initiated with a 40 sec sonication burst (unless otherwise specified) and samples were retrieved immediately after the last sonication burst for consistency. A power setting between 90% and 100% was typically used.

2.8 PMCA experimental set-up

PMCA experiments were initiated using the NBH as the substrate. Dilution series were prepared in 1.5 mL snap-cap microcentrifuge tubes (Eppendorf, Mississauga, ON), and typically consisted of 2, 5 or 10 fold dilutions of the sample into the 10% NBH stock (e.g., Table 2.2). Tubes in the dilution series were mixed by inverting (never vortexed) in an effort to avoid destroying

important components/co-factors. Eighty µL from each dilution was aliquoted into labeled 0.2 mL thin walled PCR tubes (Nalgene/Nunc International, Rochester, NY, USA, catalogue #250875). Closed sample tubes were sprayed with 2M NaOH and left at room temperature inside a biosafety cabinet for 1 hour before being removed and frozen to -80°C. All tubes, except for the -80°C unsonicated time-zero control, were removed from the freezer after 30 minutes and allowed to thaw to room temperature. The sample tubes were placed in the 37°C water bath, and immediately exposed to sonication. Multiple replicates for the variables tested were from independent experiments where the CB and NBH was prepared fresh, and the dilution series was set-up as outlined below (Table 2.2).

Table 2.2: Example of a dilution series prepared for PMCA experiment set-up.

Dilution of IBH	**10% IBH (µL)**	**10% NBH (µL)**	**Total volume (µL)**	**% IBH**
0 (NBH only)	0	80	80	0
1:10^{1}	12	108	120	1
1:10^{2}	12 of 1:10^{1}	108	120	0.1
1: 10^{3}	12 of 1:10^{2}	108	120	0.01
1: 10^{4}	12 of 1:10^{3}	108	120	0.001
1: 10^{5}	12 of 1:10^{4}	108	120	0.0001
1: 10^{6}	12 of 1:10^{5}	108	120	0.00001
1: 10^{7}	12 of 1:10^{6}	108	120	0.000001
1: 10^{8}	12 of 1:10^{7}	108	120	0.0000001
1: 10^{9}	12 of 1:10^{8}	108	120	0.00000001
1: 10^{10}	12 of 1:10^{9}	108	120	0.000000001

2.9 Adaptation, optimization and standardization of the PMCA PrP^{CWD} assay

While developing the PMCA protocol for amplification and detection of PrP^{CWD}, various modifications had to be made to the basic PMCA PrP^{Sc} protocol previously developed by members of our research team. In order to replicate past results, using different equipment inside a new facility and adapt the protocol from amplification and detection of scrapie 263K to chronic wasting disease, changes involving the detection and amplification were implemented.

2.9.1 Detection of PrP^{CWD} by Western blot

Since the 3F4 antibody used in the PMCA PrP^{Sc} protocol does not recognize any cervid species, an antibody with broader specificity had to be found. The 6H4 antibody from Prionics recognizes the sequence DYEDRYYRE of the prion protein. This sequence is conserved in most known mammalian PrP sequences and was chosen for this reason. It is believed that the PrP^{CWD} is more protease sensitive than the scrapie 263K so PrP^{CWD} Proteinase K digest susceptibility was determined by (0 - 200µg/mL) in an attempt to find a balance between removal of all the normal prion protein without the removal of any protease sensitive infectious PrP. It was hypothesized that the solubility of the PrP^{CWD} was different from that of the scrapie and changes to the Proteinase K digest were required. Detergents were added to the PK solution in order to avoid protein aggregation that could prevent protease access and lead to incomplete digestion of the normal prion protein. If bound to the 6H4 antibody, any normal prion protein would

produce a false positive signal. Strategies to recognize false positives resulting from incomplete digestion, *de novo* production and cross contamination had to also be considered. Controls for incomplete digestion included normal brain homogenate (i.e., NBH only) samples treated with PK for which a band shift was not noted. These samples also acted as controls for *de novo* production based on the presence of a protease-resistant band at the 27-30 Kd range. Multiple NBH control samples were included throughout the preparation phase to ensure no cross contamination of samples associated with aerosolization or liquid phase transfers.

2.9.2 Amplification of PrP^{CWD} by the PMCA PrP^{CWD} assay

Replicating the levels of amplification previously attained by members of our research team, in a new facility with new equipment, proved difficult. Machine-to-machine and model-to-model variation had not been encountered or evaluated and optimal power settings for each sonicator unit had to be found. Unexpected variables, such as sample position and horn degradation, when recognized, had to be adjusted for in order to produce reliable results.

2.9.3 Standardization of the PMCA PrP^{CWD} protocol

Samples (tissue, feces, urine, saliva) from experimentally and naturally infected cervids were used as the infectious source and the normal brains from TgElk cervidized mice were used for the normal brain homogenate substrate. Initially, 80uL serial dilutions of 10% (w/v) infectious CWD sample homogenate

diluted with 10% NBH (normal hamster brains perfused with cold PBS containing 5mM EDTA at sacrifice) were prepared in a conversion buffer (Saá *et al.*, 2006). PMCA cycles were carried out with the use of a Misonix 4000 programmed for cycles of 30 minutes of incubation (no sonication) followed by 40 seconds of sonication at a power setting of 90 for 37 cycles over 18 hours in a 35°C incubator. This constituted one round. The product of the first round of PMCA was then diluted 1:10 into fresh 10%NBH for a second round. Following amplification, the sample was digested with Proteinase K diluted to a final concentration of 50ug/mL in PBS with 4%SDS and 0.1% Triton X and incubated at 37°C for 20 minutes. The reaction was stopped with an equal volume of Laemmli buffer containing SDS and β-mercaptoethanol and boiled for 5 minutes. The amplified PrP^{CWD} was detected using the Western blot assay, incorporating the 6H4 antibody (1:20000) and the chemiluminescense of a goat anti-mouse horse radish peroxidase secondary antibody (1:10000) combined with Amersham's ECL (enhanced chemiluminescence) system and imaged on a GE ImageQuant 350.

2.10 Proteinase K digestion

Proteinase K (PK) digestion was carried out on PrP^{Sc} and PrP^{CWD} samples with final concentrations of 200µg PK/mL and 50ug/mL respectively. A 20mg/mL PK stock solution was prepared from the PK powder (Promega) in Milli-Q® water and frozen in 100µL aliquots. For the PMCA PrP^{Sc} samples, the PK stock solution was diluted 1:20 in Milli-Q® water. For the PMCA PrP^{CWD}

samples, the PK stock solution was diluted 1:80 in PK digest buffer (4% SDS, 0.1% Triton X-100 in 0.66x PBS). Five µL of the diluted PK stock solution was added to 20µL of experimental sample and vortexed. The PMCA PrP^{Sc} samples were incubated at 37ºC for 20 min. The PMCA PrP^{CWD} samples were incubated at 45ºC for 30 min. An equivalent volume of 2x Laemmli buffer solution (*i.e.*, 25µL) was added to reduce and denature the samples. Laemmli buffer (2x) solution was prepared according to BioRad TeSeE Purification and Detection kit; specifically, 28.5mL of Laemmli sample buffer (BioRad, Hercules, CA, catalogue #161-0737), 0.6g sodium dodecyl sulfate (SDS, Sigma-Aldrich Canada Ltd, Oakville, ON, catalogue #L4390-100G) and 1.5mL ß-mercapto-ethanol (Sigma-Aldrich Canada Ltd, Oakville, ON, catalogue #M-7154). Samples were vortexed and incubated at 97ºC for 7 min.

2.11 SDS-PAGE

Precast Pierce Precise 12% polyacrylamide gels (Fisher, catalogue # PI-25222) were placed in the electrophoresis tank (Mini-Protean Tetra Cell, BioRad, Hercules, CA, catalogue # 165-8004) and the Mini-Protean Tetra cell was filled with running buffer. Running buffer was prepared according to manufacturer's specifications (HEPES running buffer 20x, Fisher, catalogue # 28368).

Equal sample volumes (25µL) were loaded into all wells, along with one lane for 1uL of molecular weight ladder (Precision Plus Protein WesternC Standard, BioRad, Hercules, CA, USA, catalogue # 161-0385). The ladder was used to verify proper transfer from the gel to the membrane and to identify the

approximate molecular weights of the proteins. An HC Power Pac (BioRad, Hercules, CA, catalogue #164-5052) supplied the power and ran for 40 min at 130V.

2.12 Western blotting

Transfer buffer was prepared to a final concentration of 1x Tris/glycine (BioRad, Hercules, CA, USA catalogue # 161-0734 [10x stock]) and 20% methanol (Commercial Alcohols, Brampton, ON, Canada) in Milli-Q® water. Polyvinylidene difluoride membranes (PVDF, BioRad, Hercules, CA, USA, catalogue #162-0177) were cut to match the gel dimensions. To condition the membrane, they were first immersed in 100% methanol for 15 seconds then soaked in prepared transfer buffer until needed.

The SDS-PAGE (polyacrylamide gel electrophoresis) gels were carefully removed from between the plastic plates and placed in transfer buffer to equilibrate for at least 5 min prior to the transfer step. Assembly of the transfer cassettes was carried out according to the manufacturer's protocol. Disposable filter papers (BioRad, Hercules, CA, USA, catalogue #170-4085) and fiber pads were soaked in transfer buffer to minimize/eliminate the potential for air bubble intrusion during the transfer. A gel roller (BioRad, Hercules, CA, USA, catalogue #165-1279) was used to ensure no air bubbles that may have formed between the layers remained. The Trans-Blot cell (BioRad, Hercules, CA, USA, catalogue # 170-3935) was plugged into Power Pac HC (BioRad, Hercules, CA, catalogue #164-5052) and run overnight (approximately 18 hours) at 20V.

After transfer, the PVDF membrane was removed from the Trans-Blot cell and immersed in 100% methanol for 10 sec. The membrane was removed and placed on a paper towel and allowed to dry completely in order to fix the proteins to the membrane (approximately 15 minutes). Dried membranes were rewetted in 100% methanol for 5 minutes then rinsed in 1x PBS (BioRad) for another 5 minutes. The membrane was then incubated in 5% skim milk (Carnation, Nestle USA Inc, Glendale, CA) for 1 hour at room temperature, with medium agitation using a platform rocker/shaker (Fisher, catalogue # 05-450-34) to block unoccupied membrane binding sites and prevent nonspecific binding of detection antibodies.

Once the blocking solution was removed, the membranes were incubated at room temperature, under medium agitation, with primary antibody for 1 hour. For the PMCA PrP^{SC} samples, 3F4 antibody was used (Millipore, Billerica, MA, USA, catalogue #mAb1562). The 3F4 antibody binds hamster, human, cat, and baboon prion protein, and is not known to react with any other mammalian species. For the PMCA PrP^{CWD} samples, 6H4 antibody was used (Prionics, Switzerland, catalogue # 01-010). Both antibodies were diluted 1:20,000 in PBS with Tween (PBST). The PBST was prepared according to BioRad specifications with a final concentration 0.1% Tween-20 (BioRad, Hercules, CA, USA, catalogue #170-6531) in 1x PBS (BioRad, Hercules, CA, USA, catalogue #161-0780). After incubation with primary antibody, the antibody solution was poured off and the membrane washed three times with PBST for 10 minutes each.

The conjugated secondary antibody, goat anti-mouse horse-radish peroxidase (GAM-HRP, BioRad, Hercules, CA, USA, catalogue #170-6516), was

added to bind to the primary antibody at a 1:10,000 dilution. An HRP conjugate for the WesternC Standard ladder was also added to the secondary antibody solution at a 1:50000 dilution. The membrane was immersed and agitated in secondary antibody solution for 1 hour. The membrane was washed 3x in PBST as before and then rinsed twice with 1x PBS (BioRad) to eliminate as much detergent (*i.e.,* Tween) as possible. To validate the specificity of secondary antibody binding, the primary antibody step was eliminated in some experiments.

Chemiluminescent detection was performed using the ECL Plus reagent (Amersham, GE Life Sciences, Canada, catalogue #RPN2109) according to manufacturer's instructions. After seven minutes, excess ECL reagent was removed by dabbing the corner of the membrane to absorbent paper. The membrane was placed into a plastic ziplock bag and passed outside of the Level 3 biocontainment laboratory through a dunk tank containing 5% virkon. The bag containing the membrane was retrieved on the other side of containment, rinsed off with regular tap water, placed inside an ImageQuant 350 (GE Healthcare, catalogue # 167343) and under the chemiluminescent setting, exposed for three minutes. All Western blot images were then exported as jpeg images for analysis.

2.13 Analysis of PMCA results

Western blot images were used to calculate the amount of amplification produced by the PMCA by visually comparing the WB pattern (dilution series) of the post-PMCA dilution series to the -80ºC frozen controls that were not subjected to sonication. By counting the number of dilutions where a PrP^{Res} band was

observed and using this number as the exponent to the base dilution factor, in this case 10, amplification could be approximated. For example, if three dilutions in the series displayed bands after PMCA, and the final dilution yielded a band of equal visual intensity to those observed in the -80ºC frozen control, the calculated amplification rate would be 10^3, *i.e.*, 1000x. If amplification was observed in the lowest dilution of the series, this dilution with a 'greater than' value was used for calculation purposes. For average amplification calculation, absolute values of amplification were used and the result rounded to one significant figure. In experiments where sPMCA was employed, the dilution between rounds of PMCA was included in the calculation of average amplification. For instance if two rounds of PMCA were performed with a 1:10 dilution between rounds the resulting average amplification was multiplied by the dilution factor (*i.e.*, 10).

CHAPTER 3 ADAPTATION, OPTIMIZATION AND STANDARDIZATION OF THE PMCA ASSAY FOR DETECTION OF PrP^{CWD}

3.1 Introduction

Prior to 2009, when this thesis was initiated, relatively few laboratories had reported on using PMCA for detection of CWD. Kurt et al (2008) demonstrated the advantage of transgenic mice [Tg(cerPrP)1536] over normal cervid brain as a source of PrP^{C} for amplification, and Green et al (2008) using the same transgenic mouse line, showed high fidelity amplification of CWD prions with unaltered properties. Meyerett et al (2008) demonstrated that PMCA can amplify and adapt prion strains *in vitro* as effectively and much more quickly than *in vivo* strain adaption by mouse passage. In all three studies both the PMCA and Western Blot (i.e., PK digest) conditions were different.

Over the last five years, members of our research team have been involved in the development of a PMCA assay for the detection of prions in a 263K hamster scrapie model, with this acting as the basis for development of the PMCA assay for CWD. The original technical publication of Castilla *et al.* (2006), outlined the PMCA protocol and its parameters, and served as the primary resource for the scrapie PMCA assay. Five deviations from the original publication by Castilla (2006) were incorporated into the 263K PMCA assay that was developed in our laboratory (Braithwaite, 2009): 1) lower centrifugation (speed and time) of the normal brain homogenate, 2) the addition of EDTA to the conversion buffer, 3) decreased duration of the PMCA cycle, 4) lower sonication intensity and 5) the addition of heparin as a facilitator of PMCA for 263K scrapie.

The first two changes were to err on the side of caution based on recommendations that high centrifugal force might remove important membrane components that act as conversion factors necessary for PrP^{C} to misfold into PrP^{Sc}(Saá *et al.*, 2005), along with the successful reports of amplification with EDTA in the conversion buffer preparation by both Castilla *et al.* (Castilla *et al.*, 2005) and Saa *et al.* (Saá *et al.*, 2005). Using the basic 24 hour (48 cycles) PMCA protocol, PrP^{Sc} was reproducibly amplified *in vitro* by approximately 5x. Unfortunately this level of amplification was lower than all reports in the literature (Castilla *et al.*, 2005; Lucassen *et al.*, 2003; Saá *et al.*, 2006; Saborio *et al.*, 2001). Amplification was improved through optimization of variables such as duration and sonication intensity. The optimal length of time was determined to be 16 hours based on the higher average amplification, as well as providing more consistent and reproducible results (Braithwaite, 2009). From a laboratory standpoint the overnight duration was convenient and logical; PMCA samples could be set-up and placed in the sonicator at the end of the day and retrieved the following morning and processed. Sonication intensity had previously been reported to be optimal for 263K at a potency of 80-100% for PMCA systems (Atarashi *et al.*, 2007; Castilla *et al.*, 2006; Saá *et al.*, 2005). Castilla *et al* (Castilla *et al.*, 2006) suggested that an increase in sonication intensity may be required if the number of samples exceeded 60% of the sample rack capacity and that after extended use the sonicator intensity may diminish over time (Deleault *et al.*, 2007). A power setting of 60% was found to be optimal for the PMCA PrP^{Sc} assay using the 263K hamster scrapie model. The addition of hamster plasma that had been collected with a heparin anticoagulant optimized the PMCA assay to

yield a 5-6 log amplification of PrP^{Sc} within two 16 hour rounds of PMCA, representing a significant improvement in previously reported PMCA protocols (Saá *et al.*, 2005; Saá *et al.*, 2006). Subsequent work in our laboratory has demonstrated a significantly greater level of amplification when heparin is used during the amplification of scrapie 263K by PMCA (Braithwaite *et al.*, manuscript submitted).

This modified, and optimized protocol for amplification of PrP^{Sc} for 263K hamster scrapie acted as the basis for developing a PMCA assay for PrP^{CWD}. The objective of this chapter of the thesis was to develop a robust procedure for detecting PrP^{CWD} using PMCA.

3.2 Results and Discussion

Developmental work on the PrP^{CWD} PMCA assay was performed in a new Level 3 biocontainment facility (Alberta Agriculture and Rural Development). This work required adaptation and re-optimization of the basic scrapie 263K PMCA assay on new sonicators located within this facility, and therefore the initial work on this thesis focused on ensuring PMCA amplification of 263K scrapie, followed by adaptation of the assay conditions for amplification of PrP^{CWD}.

3.2.1. Validation and Optimization of PMCA Efficiency Using Scrapie 263K

Adjustments to the basic scrapie PMCA protocol included increasing the cycle duration to 18 hours (due to ease of access and work hour limitations within

the Level III facility), increasing sonication intensity to 90%, and re-assessing position placement of samples within modified sonicator models as discrepancies in PMCA efficiency were noted based on positional placements of the individual tubes within the sonicator horn. Using a tube rack provided for the water bath/sonicator horn configuration, optimal sample tube arrangement over the sonicating horn had to be established (Fig. 3.1).

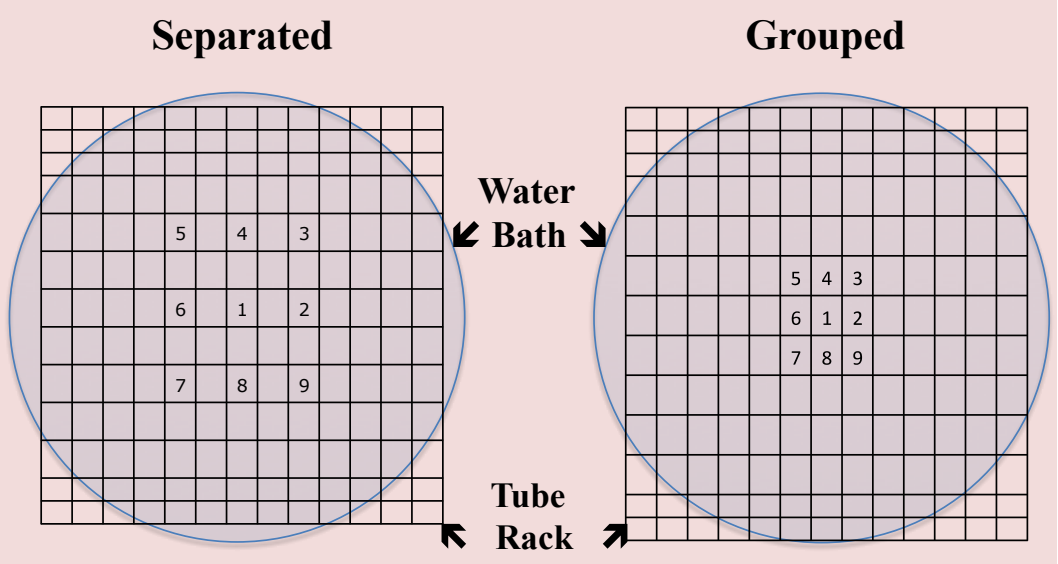

FIGURE 3.1: POSITION OF SAMPLES INSIDE THE SONICATOR.

A direct comparison was performed to determine whether a separated or grouped arrangement of the tubes was more efficient. Scrapie 263K infectious brain homogenate was serially diluted into 10% normal hamster brain homogenate (w/v) and subjected to one round of PMCA amplification at a power setting of 90% for 18 cycles consisting of a 40 second pulse every half hour for both sets of tube configurations in parallel.

The separated sample arrangement (Fig. 3.2 Panel A) was able to detect the 263K scrapie down to a 1:312500 dilution, while the set that was centrally grouped together (Fig. 3.2 Panel B) detected PrP^{Sc} down to the maximum dilution of 1:7812500 suggesting that further dilutions could have been accommodated. A central arrangement was considered more effective for amplification and incorporated into the protocol.

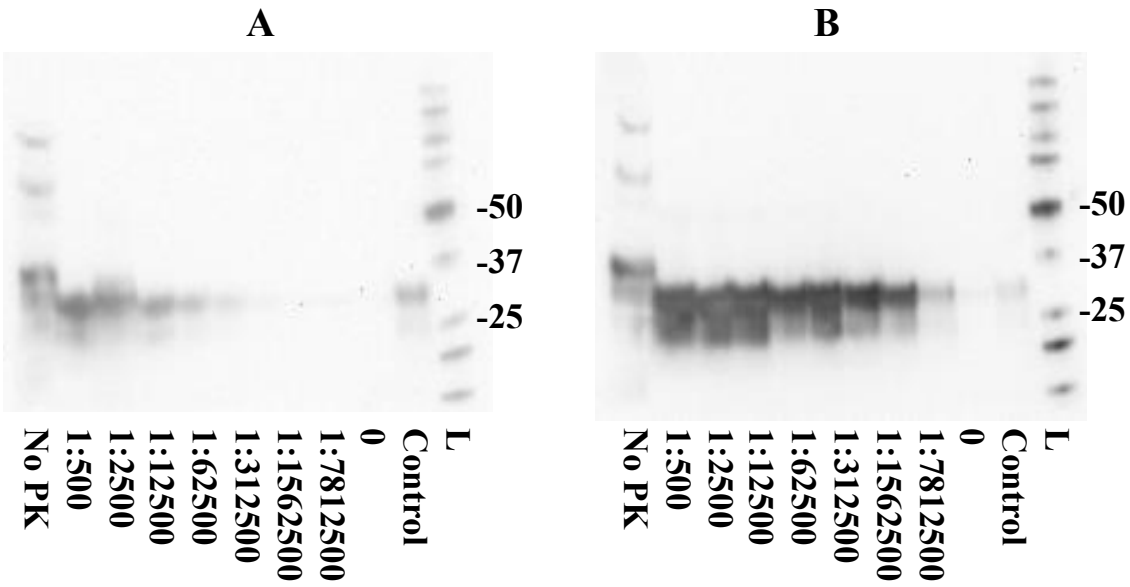

FIGURE 3.2: WESTERN BLOTS DEMONSTRATING INCREASED AMPLIFICATION BASED ON SAMPLE POSITION ACROSS THE SONICATOR HORN.

PMCA was performed with samples either separated (Panel A) or grouped together in the center of the sonicator horn (Panel B) based on Figure 3.2. Scrapie 263K infectious brain homogenate was serially diluted into 10% normal hamster brain homogenate (labeled 1:500 – 1:7812500 and 0), placed into positions 1 to 9 respectively and subjected to 1 round of PMCA. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{Sc} seed), 'Control' – 1:10 dilution of a 10% IBH sample stored at -80^{o}C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

3.2.2. Validation and Optimization of PMCA Efficiency for CWD

Once re-optimization of the hamster 263K PMCA was performed, this basic protocol was used in an attempt to detect and amplify PrP^{CWD} by PMCA, but with three specific changes. Firstly, the antibody 3F4 was substituted for the antibody 6H4 during the Western blot procedure because of its broader specificity to detect prion protein in mammals other than human and hamster. Secondly, the source of normal cervid PrP^{C} for the PMCA (i.e., NBH) was prepared from transgenic mice containing the elk prion protein [TgElk, (LaFauci *et al.*, 2006)]. Previous work by other groups adapting PMCA to detection of PrP^{CWD} demonstrated the lack of amplification efficiency in the PMCA reaction when deer brains were used as a source of PrP^{C} (Kurt *et al.*, 2007). No previous studies had reported the efficiency of using TgElk brains for detection of PrP^{CWD} by PMCA. Thirdly, since both 3F4 and 6H4 antibodies detect the normal and infectious form of the prion protein (PrP^{C} and PrP^{Sc}), specific detection of PrP^{Sc} was dependent on the use of a Proteinase K digest to differentiate between the two isoforms. The PK digest concentration and conditions were modified, as data from the literature suggested a lower PK sensitivity threshold for CWD compared to 263K scrapie and often performed under different digest buffer conditions (Kurt *et al.*, 2007; Meyerett *et al.*, 2008; Haley *et al.*, 2009a).

In addition, optimization of the PMCA assay focused on detection of PrP^{CWD} from two sources: a) a wildtype strain of CWD that originated from an infected WTD farm ($PrP^{CWD/WTD}$) and b) CWD originating from an

experimentally-infected elk ($PrP^{CWD/Elk)}$. Although the $PrP^{CWD/Elk}$ strain was used to adapt and optimize the detection of CWD, it was important to ensure that the PMCA assay was also capable of efficiently detecting the $PrP^{CWD/WTD}$ strain, as it was this strain that was used to experimentally infect WTD at the CFIA, and for which the course of disease in WTD was monitored using PMCA for detection of PrP^{CWD} in these animals (as described in Chapter 4 of this thesis).

3.2.2.1 Proteinase K digest conditions

Even with a lower Proteinase K concentration for the digestion of PrP^{C} and a more appropriate antibody (6H4), initial detection of PrP^{CWD} in the white-tailed deer infectious brain homogenate proved difficult with no Western blot signal found at any dilution (Fig. 3.3).

However, verification of the presence of PrP^{CWD} in these samples was performed at the CFIA using a BioRad TSE detection kit (Balachandran, personal communication). The BioRad kit incorporates a concentration step prior to detection by Western blot possibly explaining the discrepancies between sensitivity levels observed by the kit and direct detection of PrP^{CWD} by Western blotting of crude brain homogenates. Consequently, this result required a slightly different approach to method optimization of PMCA for CWD. Both amplification and PK detection sensitivities for WTD CWD were optimized simultaneously, with PMCA performed using TgElk brain homogenates as a

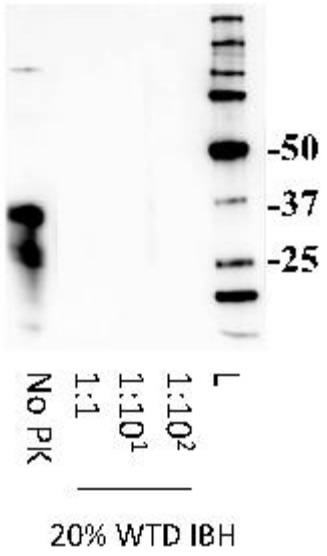

FIGURE 3.3: WESTERN BLOT DEMONSTRATING INABILITY TO DETECT PRP^{CWD} IN THE CRUDE WHITE-TAILED DEER INFECTIOUS BRAIN HOMOGENATE FROM A WILDTYPE STRAIN OF CWD THAT ORIGINATED FROM AN INFECTED WTD FARM PROVIDED BY THE CFIA.

A 20% WTD IBH was serially diluted into 10% TgElk normal brain homogenate (labeled 1:1 – 1:100). Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

source of cervid PrP^{C} for amplification, and in some cases CWD from experimentally-infected elk (provided by Dr. Catherine Graham, CFIA) were used as a seeding source for the PMCA and which was readily detectable in 20% IBH samples by standard Western blots (i.e., not requiring the BioRad Kit). Several variables involved in the Proteinase K (PK) digestion had to be optimized for the PMCA PrP^{CWD} assay, including: 1) final concentrations of PK for digestion of PrP^{C}, 2) PK digest temperature 3) PK buffer solution and 4) PK digest time.

3.2.2.1.1 Proteinase K final concentration

A dilution series ranging from 10ug/mL to 220ug/mL of Proteinase K was used to digest a 10% white-tailed deer normal brain homogenate (w/v) at 37°C for 30 minutes (Fig. 3.4).

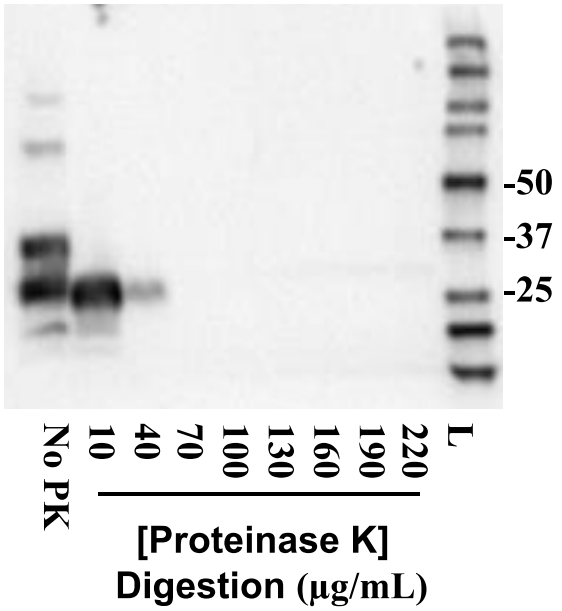

FIGURE 3.4: WESTERN BLOT DEMONSTRATING THE EFFECT OF PROTEINASE K CONCENTRATION ON THE DIGESTION OF WTD NORMAL BRAIN HOMOGENATE.

A dilution series ranging from 10µg/mL to 220µg/mL of Proteinase K in dH_2O was used to digest 10% white-tail deer normal brain homogenate at 37°C for 30 minutes. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

A strong band within the Western blot was observed in the 10ug/mL digested sample and a weak band was detected in the 40ug/mL, indicating that 40ug/mL or lower of PK was not sufficient for complete digestion of PrP^C found in normal

brain homogenates. No bands were detected for any of the other PK concentrations suggesting that complete digestion of PrP^{C} had occurred.

Various reports have suggested that although the abnormally-folded prion protein is resistant to PK digestion, as much as 90% of PrP^{Sc} may be protease sensitive (Safar *et al.*, 1998). Furthermore, extended digestion periods are also known result in a decrease in sensitivity of detection of PrP^{Sc} over background levels of PrP^{C} (Safar *et al.*, 1998). In order to find an optimal threshold level for digesting all of the PrP^{C} without digesting too much of the PrP^{CWD}, a 5-fold dilution series of 10% WTD infectious brain homogenate (IBH) into 10% WTD NBH was set up (Fig. 3.5) and subjected to PMCA sonication, to determine both effectiveness of PK digestion conditions and ability of WTD brain homogenates to act as a source of cervid PrP^{C} for amplification of PrP^{CWD}.

No signal was detected in any of the digested samples indicating that all the detectable PrP^{C} in normal brain homogenate had been digested but no apparent amplification had occurred when WTD normal brain homogenates were used as a source of PrP^{C} for amplification (Fig. 3.5). In addition, the control (i.e., WTD infectious brain homogenate stored at -80^{o}C and subsequently digested with PK) was also blank indicating that at a 1:5 dilution of the infectious brain homogenate, the WB was insensitive at detecting PrP^{CWD} in this crude homogenate sample (as described above).

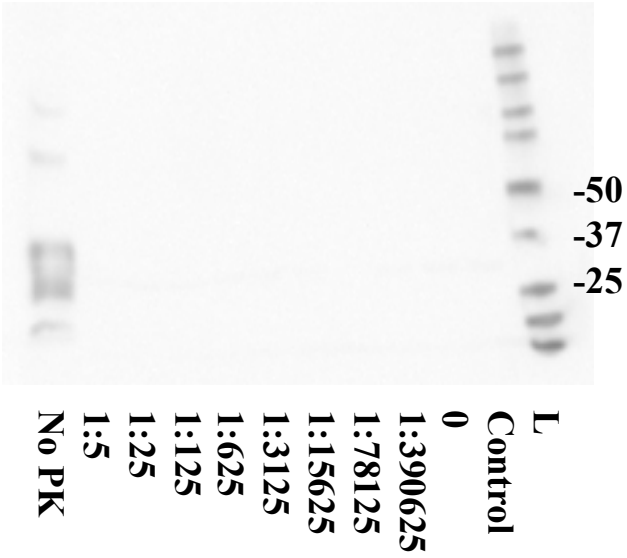

FIGURE 3.5: WESTERN BLOT DEMONSTRATING THE EFFECT OF THE PK DIGESTION AT 50µG/ML H_2O ON 10% WHITE TAIL DEER (WTD) NORMAL BRAIN HOMOGENATE (NBH) SPIKED WITH A WTD INFECTIOUS BRAIN HOMOGENATE (IBH) AT DIFFERENT DILUTIONS.

A five-fold dilution series of 10% WTD IBH was diluted into 10% WTD NBH and amplified by PMCA. PMCA conditions were as follows: 90% power, 40 second sonication pulses every 30 minutes for 18 hours. Other lanes are designated as follows:, 'No PK' – NBH not treated with Proteinase K; '0' – NBH only (no PrP^{CWD} seed); 'Control' – 1:5 dilution of a 10% WTD IBH sample stored at -80^{o}C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown)

3.2.2.1.2 Proteinase K digest temperature

The temperature of PK digestion was addressed in defining the optimal conditions for detection of PrP^{CWD}. Due to the inability to detect $PrP^{CWD/WTD}$ in the crude brain homogenates from an infected WTD, a sample of $PrP^{CWD/Elk}$ was used to further optimize PK digests temperatures and assess amplification efficiency. Samples from a 5-fold dilution series of a 20% elk infectious brain homogenate (IBH) diluted into 10% TgElk NBH were sonicated at 90% for 37

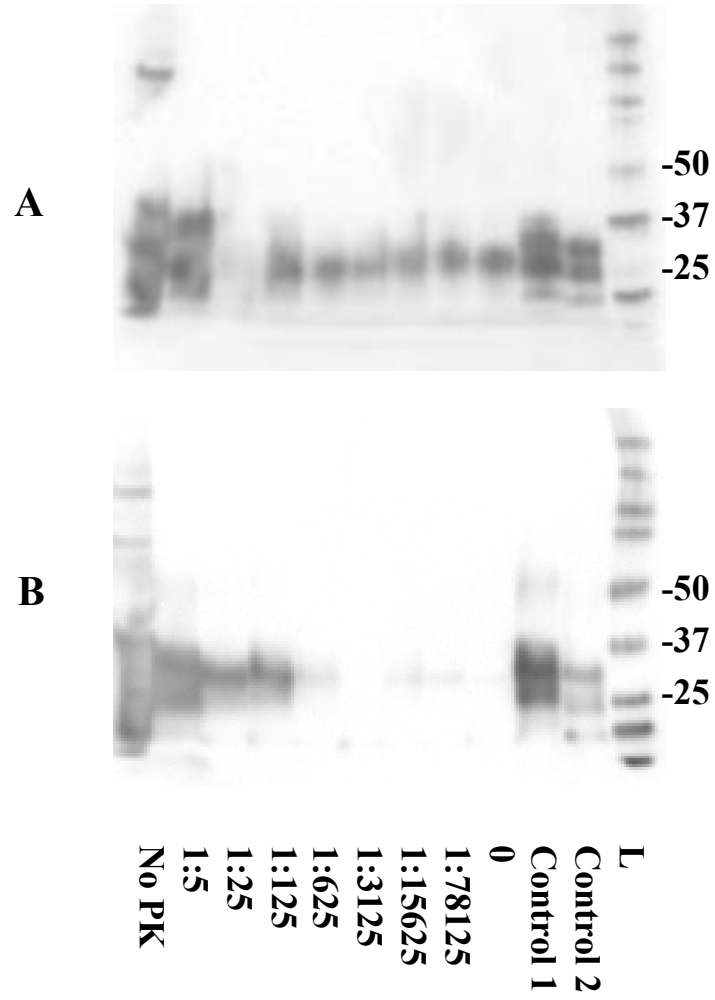

FIGURE 3.6: WESTERN BLOTS DEMONSTRATING THE EFFECT OF DIGEST TEMPERATURE.

Parallel samples of a 10% elk infectious brain homogenate serially diluted into 10% TgElk normal brain homogenate (labeled 1:5 – 1:78,125) were subjected to 1 round of PMCA and treated with Proteinase K (50ug/mL for 30 minutes) at either 37°C (Panel A) or 45°C (Panel B). Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed), 'Control 1' – 1:5 dilution of a 10% elk IBH sample stored at -80^{o}C and not subjected to PMCA, 'Control 2' – 1:5 dilution of a 10% elk IBH sample into conversion buffer, stored at -80^{o}C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

cycles, split into two sets and digested in parallel (Fig. 3.6), using 50ug/mL PK in a buffer solution for 30 minutes at either 37°C or 45°C. A significant difference was detected between the two sets of conditions, with 45°C being more effective than 37°C in digesting all PrP^{C} within the sample.

Incomplete digestion was observed in the 37°C sample, as assessed by a band in the unspiked control (i.e., NBH containing PrP^{C} only) and this same band persisting in all other samples. There was no observable tapering of band intensity associated with dilutions of IBH used to spike the PMCA reaction. The results of the second set, digested at 45°C (Fig. 3.6, Panel B), resulted in no band in the unspiked control, with a consistent banding pattern and a tapered amplification pattern fading out at a PrP^{CWD} seeding dilution of 1:625.

3.2.2.1.3 Proteinase K buffer solution

Based on several previous publications (Haley *et al.*, 2009a; Haley *et al.*, 2009b), combinations of Triton X and SDS were used in conjunction with Proteinase K (PK) digest in order to achieve complete digestion of the normal PrP but retain detection sensitivities of the PrP^{CWD} isotype. For the 263K scrapie assay, the PK stock solution had been prepared in distilled water at 20mg/mL and frozen in 100ul aliquots. The PK stock solution was then diluted to 1mg/mL in distilled water and added to the sample at a ratio of 1:5, typically 10ul of 1mg/mL PK to 40ul of sample homogenate for a final digest concentration of 200ug/mL. A similar approach was used to evaluate PK digest of CWD, but with a final PK concentration of 50ug/mL in water alone, or containing Triton-X and SDS.

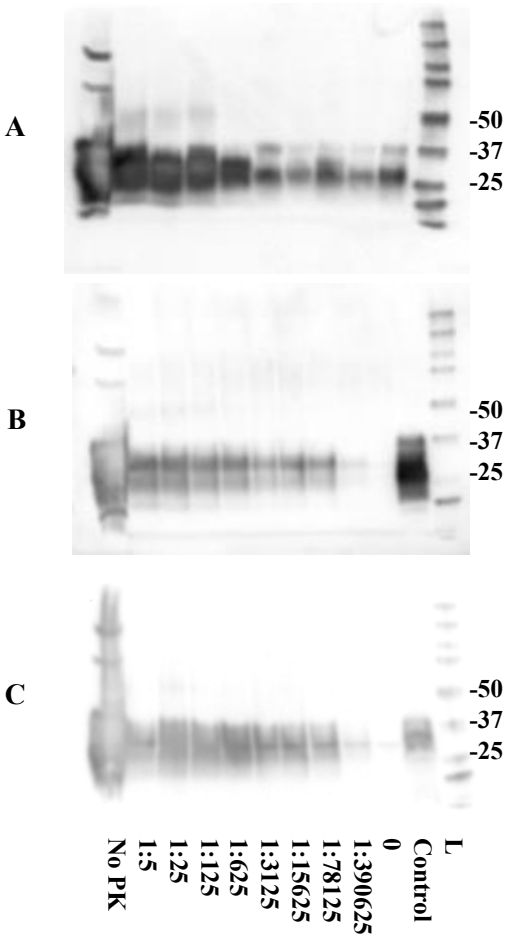

FIGURE 3.7: WESTERN BLOTS DEMONSTRATING THE EFFECT OF BUFFER CONDITIONS ON PROTEINASE K (PK) DIGEST OF PRP^{CWD}.

A 20% elk infectious brain homogenate (IBH) was serially diluted (labeled as 1:5 – 1:390,625) into 10% TgElk normal brain homogenate and subjected to 1 round of PMCA at a power setting of 90%. To test the effect of detergent on the PK digest, a 20mg/mL PK stock solution made by reconstituting lyophilized PK powder in distilled H_2O (dH_2O) was diluted in three solutions: water (Panel A), 0.035% Triton X and 1.4% SDS (Panel B) or 0.02% Triton X and 0.8% SDS (Panel C). The samples were digested at 45°C for 30 minutes. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed), 'Control' – 1:5 dilution of a 10% elk IBH sample stored at -80°C and not subjected to PMCA and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

Unlike 263K scrapie, PK diluted in water alone resulted in the incomplete digestion of PrP^{C} as demonstrated by the presences of several bands in negative controls where no infectious PrP^{CWD} was added (Fig. 3.7, Panel A). It was hypothesized that solubility issues associated with the prion protein in water may cause aggregation of lipophilic molecules, thereby trapping PrP^{C} and not allowing full access of the PK to all protein molecules in solution. This incomplete digestion would consequently allow the 6H4 antibody to bind to leftover normal PrP, producing a false detectable signal by Western blot.

The presence of Triton X and SDS within the PK digest solution resolved the issue of incomplete digestion of PrP^{C} observed when PK was diluted in water only (Fig. 3.7). In this context, a final concentration of 0.02% Triton X and 0.8% SDS in the PK buffer solution was selected as the optimal conditions for PK digestion of cervid PrP^{C} with subsequent detection of PrP^{CWD} by Western Blot (Fig. 3.7).

The results from Figure 3.8 also demonstrate a relatively high level of amplification of PrP^{CWD} associated with the PMCA assay under the experimental conditions described. In this experiment, a 20% IBH obtained from an experimentally-infected elk could be diluted 1:78,125 fold and still be detected by PMCA (compared to unspiked controls) suggesting an approximate 4 $\log_{10}$ amplification of PrP^{CWD} during 1 round of PMCA. The data also demonstrated the utility of using TgElk mouse brains as a source of PrP^{C} for amplification of PrP^{CWD} by PMCA.

3.2.2.1.4 Duration of Proteinase K digestion

The duration of PK digestion was also addressed in defining the optimal conditions for detection of PrP^{CWD} (Fig. 3.8). Given the successful amplification of $PrP^{CWD/Elk}$ using TgElk NBH as a substrate for amplification, samples from a 5-fold dilution series of 10% WTD infectious brain homogenate (IBH) diluted into

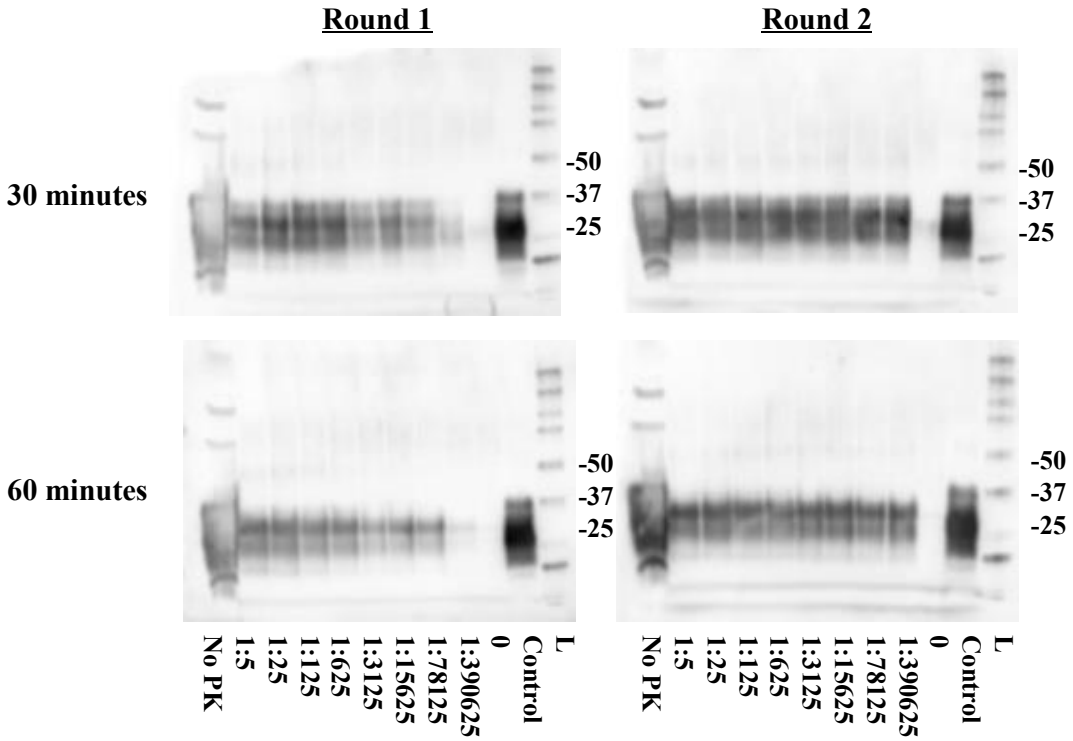

FIGURE 3.8: WESTERN BLOTS DEMONSTRATING THE EFFECT OF DIGESTION TIME ON IDENTICAL SAMPLES AFTER SPMCA SONICATION.

A 10% elk infectious brain homogenate was serially diluted into 10% TgElk normal brain homogenate (labeled 1:5 – 1:390625), subjected to 2 rounds of PMCA and treated with 50µg/mL Proteinase K (PK) for either 30 or 60 minutes. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed), 'Control' – 1:5 dilution of a 10% elk IBH sample stored at -80^{o}C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

10% TgElk NBH was subjected to PMCA, split into two sets and digested in parallel, with one set digested for 30 minutes and the second set for 60 minutes. No significant difference was detected between the two sets indicating that a half hour digest time was sufficient in digesting all PrP^{C} within the samples.

Similar to what was observed in Figure 3.7, a single round of PMCA resulted in an approximately 4 $\log_{10}$ level of amplification of $PrP^{CWD/WTD}$ (Fig. 3.8). Diluting the primary PMCA product 1:10 into fresh TgElk NBH for a second round of PMCA resulted in a >5 $\log_{10}$ amplication of $PrP^{CWD/WTD}$ (Fig. 3.8).

3.2.2.2 Evaluation of Criteria for PMCA

Overall, an evaluation of the success of PMCA for amplification of PrP^{CWD} was based on the following criteria: 1) no observable bands in unspiked controls, suggesting complete digestion of PrP^{C} during PK digestion, 2) evidence of a tapered amplification pattern, consistent with the dilution of PrP^{CWD} used to spike the reaction, 3) evidence of additional amplification of PrP^{CWD} in subsequent rounds (i.e., during serial PMCA), 4) and the quality of the band image itself. These strategies were used to: a) ensure complete digestion of PrP^{C} thereby reducing errors associated with false positive reactions, b) characterize the possible *de novo* production of PrP^{CWD} (discussed below) as reported to occur during PMCA (Deleault *et al.*, 2007), and c) evaluate cross contamination through experimental error.

3.2.2.3 Amplification of PrP^{CWD}

Once the conditions for optimal detection of the PrP^{CWD} by Western blot had been established, conditions to maximize amplification efficiency were explored. Two variables seemed to have to greatest impact on whether amplification would happen and to what extent it would occur: 1) the sonicator power setting and 2) the sample's position inside the sonicator.

3.2.2.3.1 Sonicator power setting for CWD

Members of our research team working on the scrapie PMCA assay found that a power setting of 6 was optimal for amplification of hamster 263K scrapie. When the equipment was set up in the new Level 3 biocontainment facility, the same (or identical) model of sonicator was no longer available for purchase, and a new model of sonicator was made available (Misonix Sonicator 4000). The new model no longer had a power setting range of 1 to 10 but instead the power ranged from 1% to 100%. It was presumed that a setting of 6 on the old model would be the equivalent of 60% on the new model, and consequently, power settings of 10%, 60% and 90% were chosen for initial optimization of PMCA for CWD on the new sonicators (Fig. 3.9).

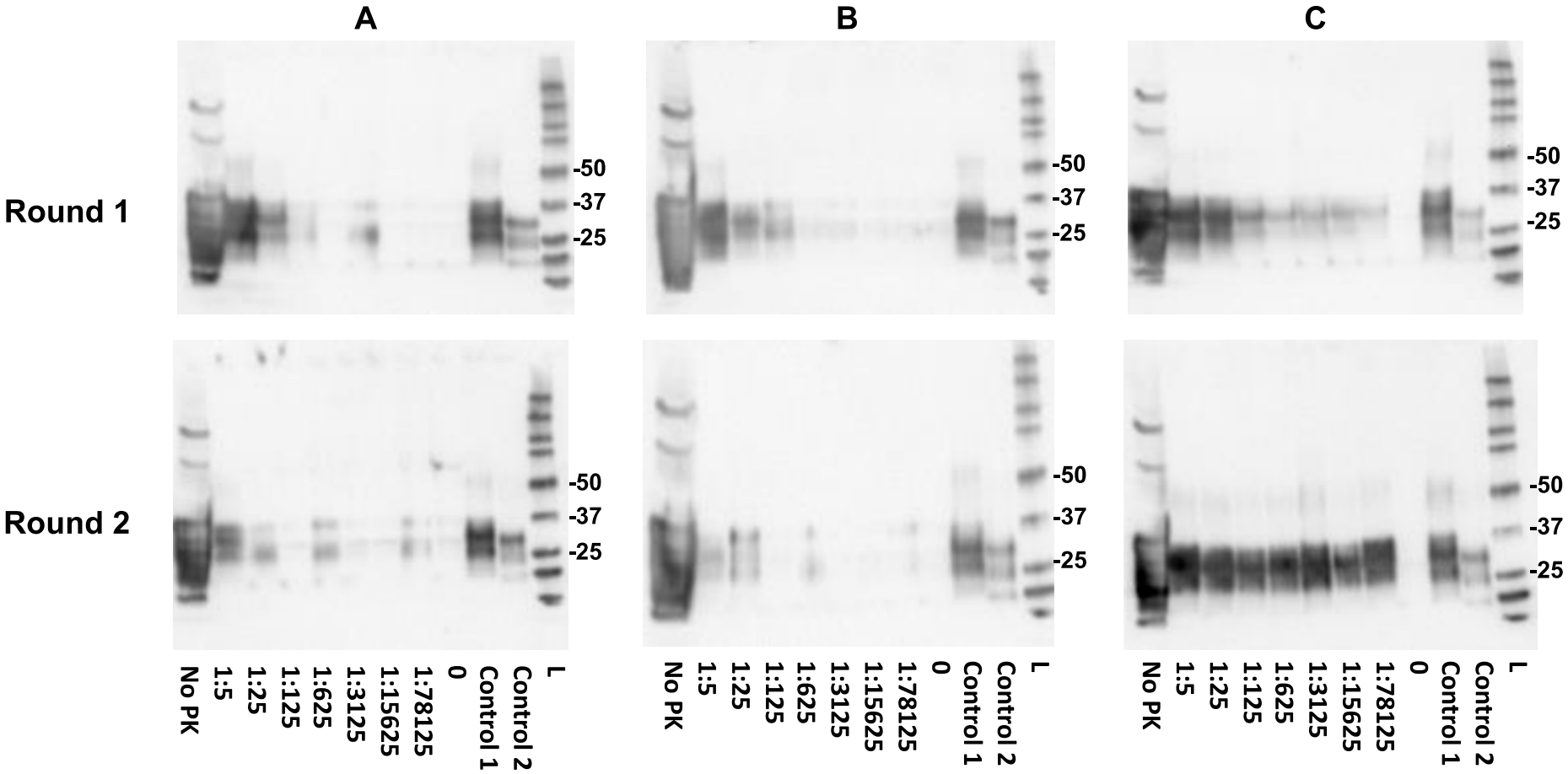
A
B
C
Round 1
Round 2
-50
-37
-25
No PK
1:5
1:25
1:125
1:625
1:3125
1:15625
1:78125
0
Control 1
Control 2
L

FIGURE 3.9: WESTERN BLOTS DEMONSTRATING AMPLIFICATION AT LOW, MEDIUM AND HIGH POWER SONICATION SETTINGS.

A 20% elk infectious brain homogenate was serially diluted into 10% TgElk normal brain homogenate (labeled 1:5 – 1:78125) and subjected to 2 rounds of PMCA at power settings 10% (Panel A), 60% (Panel B) and 90% (Panel C) and treated with Proteinase K (50ug/mL for 30 minutes at 45°C). Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed), 'Control 1' – 1:5 dilution of a 10% IBH sample stored at -80°C and not subjected to PMCA, 'Control 2' – 1:5 dilution of a 10% elk IBH sample into conversion buffer, stored at -80°C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

Low and medium power settings of 10% and 60% resulted in little or no amplification of PrP^{CWD} by PMCA (Fig. 3.9 Panel A and B). Western blot signals were detected in the first three dilutions (1:5, 1:25 and 1:125), of the first round of PMCA at 10% and 60% power conditions, but the band intensities for the dilution series, when compared to the unsonicated -80°C control, suggested little amplification occurred. Conversely, PrP^{CWD} was still detected at a dilution of 1:78,125 with a power setting of 90% (Fig. 3.9 Panel C). In addition, a second round of PMCA increased signal intensity in the 90% power setting conditions, whereas no increase in signal intensity was observed in the 10% and 60% power setting conditions. When compared to the -80°C control, the 90% power setting showed a >4 $\log_{10}$ increase through the second round.

A power setting of 90% for amplification of PrP^{CWD} the elk infectious brain homogenate would consistently produce 4-5 $\log_{10}$ of amplification in the first round and 6-7 $\log_{10}$ of amplification by the second round. The amplification of PrP^{CWD} white tailed deer infectious brain homogenate at the same power would

consistently produce 3-4 $\log_{10}$ of amplification in the first round and 5-6 $\log_{10}$ of amplification by the second round. The data is consistent when considering the different levels of PrP^{CWD} within each of sample preparations (i.e., 20% elk IBH readily detectable by Western blot from crude brain homogenates whereas the WTD strain of CWD was not detectable by standard Western blotting). Moreover, after about nine months of daily use of sonicators, a reduction of 1-2 $\log_{10}$ of PrP^{CWD} amplification in the first round (and another 1-2 $\log_{10}$ in the second round) was noted. This observation is similar to that reported by Castilla *et al.* (2006) who suggested that an increase in sonication intensity might be required after extended use of the sonicator to maintain amplification efficiency by PMCA. Based on these results and reports a power setting of 95% was considered optimal for amplification of PrP^{CWD} by PMCA.

3.2.2.3.2 Positional placement in sonicators

To test whether sample position inside the sonicator affected the amplification rate, 64 identical samples consisting of a 1:1000 dilution of 10% white tailed deer infectious brain homogenate into 10% TgElk normal brain homogenate were arranged in an 8x8 grid pattern and amplified at the same rate for two rounds of PMCA. Detection of PrP^{CWD} was done according to the optimized conditions and band intensity compared based on positional placement of tubes within the sonicator (Fig. 3.10).

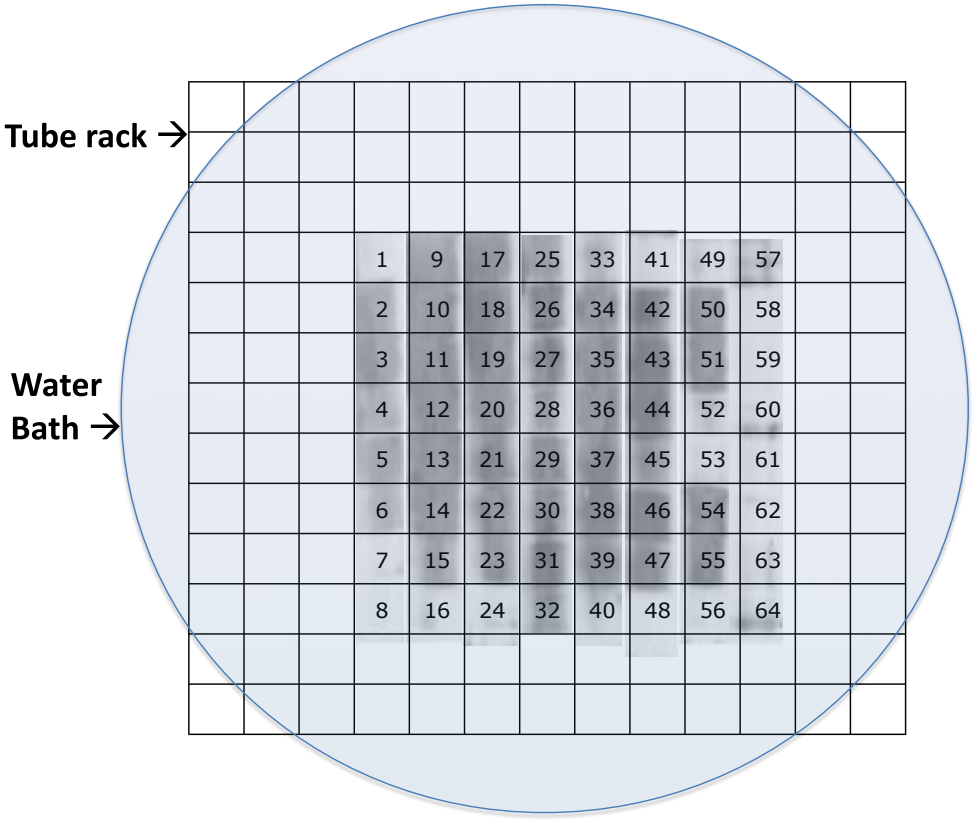

FIGURE 3.10: WESTERN BLOT IMAGES OF AN INFECTIOUS BRAIN HOMOGENATE SUBJECTED TO PMCA AND ARRANGED TO REPRESENT SAMPLE POSITION INSIDE THE WATER BATH OVER THE SONICATOR HORN.

Identical samples consisting of a 1:1000 dilution of 10% white tailed deer infectious brain homogenate into 10% TgElk normal brain homogenate were subjected to 2 rounds of PMCA at a power setting of 95% and treated with Proteinase K (50ug/mL for 30 minutes at 45°C).

A clear zone of amplification consistency could be observed in samples closest to the center of the sonicator horn and producing the strongest banding intensity. Samples farther out from this central core produced either no signal or a signal whose intensity was consistent with little or no amplification. It was concluded that no more than 30 samples placed as close to the center as possible (i.e., as defined by the vertices position of tubes 10 - 42 on the horizontal axis and

tubes 10 - 15 in the vertical axis of Fig. 3.10) would be run for each experiment in order to stay within the optimal zone of amplification.

3.2.2.4 Serial PMCA and *de novo* production of PrP^{CWD} during PMCA

Increasing the number of rounds of PMCA resulted in greater sensitivity of PrP^{CWD} by PMCA. However, on several occasions a PrP^{CWD} band could be observed in unseeded control samples after several rounds of PMCA, and usually in experiments where >3 rounds of PMCA were used (Fig. 3.11). When incomplete digestion failed to explain the presence of a band where no signal was expected, *de novo* production of PrP^{CWD} had to be considered. *De novo* production of PrP^{Sc} during PMCA has been described in several reports (Deleault *et al.*, 2007; Barria *et al.*, 2009) and three current hypotheses may explain this phenomenon; a) PrP^{Sc} *spontaneously* forms from PrP^{C} as a result of the sonication process, the product of which can seed subsequent cycles/rounds of PMCA; b) the ultra-sensitive level of detection of PrP^{Sc} by PMCA (i.e., at a 10^{-12} dilution of a 10% infectious hamster brain homogenate PrP^{Sc} can be readily detected by PMCA in our laboratory) suggests that naturally occurring misfolded proteins may exist at extremely low concentrations in all brain homogenate preparations, and consequently are amplified throughout multiple cycles/rounds; and c) like PCR, PMCA may amplify extremely small amounts of PrP^{Sc} as a result of experimental contamination (i.e., aerosolization) during experimental set up. In order to minimize these 'false positive reactions', the PMCA assay was limited to 4 rounds (or less) in most cases.

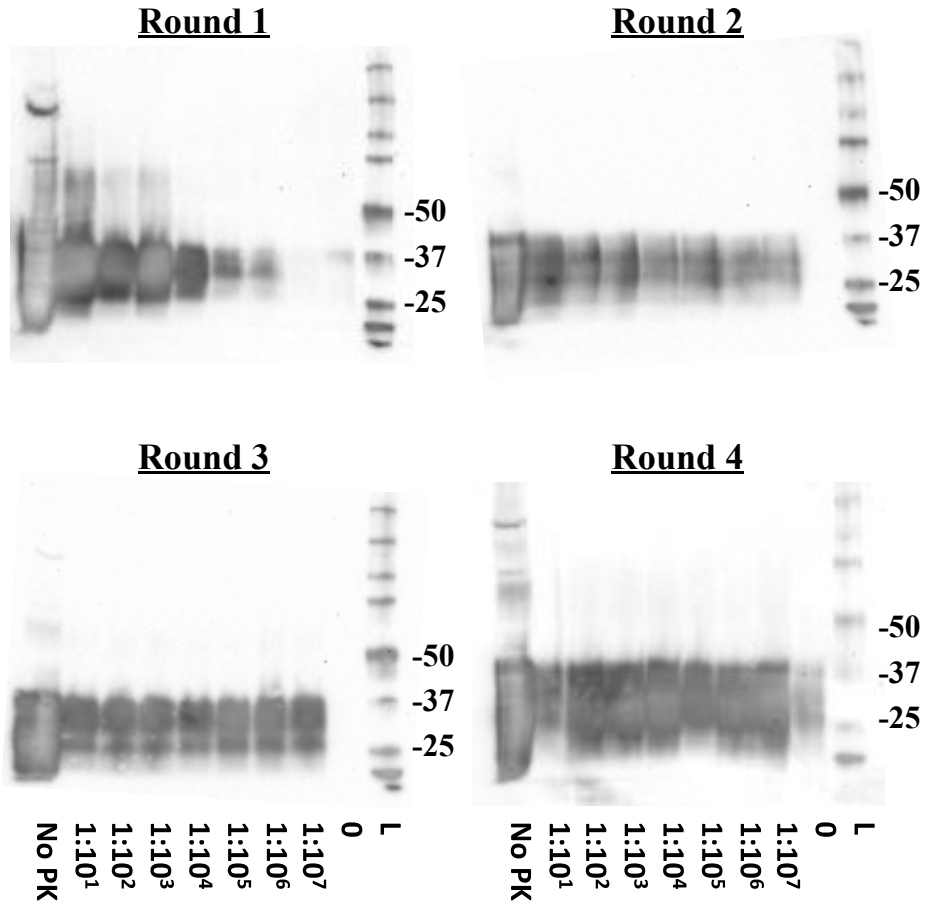

FIGURE 3.11: WESTERN BLOTS DEMONSTRATING FALSE POSITIVE DETECTION OF PRP^{CWD} THROUGH POSSIBLE DE NOVO PRODUCTION.

A 10% mule deer infectious brain homogenate serially diluted into 10% TgElk normal brain homogenate (labeled 1:10^1 – 1:10^7) was subjected to PMCA and treated with Proteinase K (50ug/mL for 30 minutes at 45°C) over 4 rounds (A [Round 1], B [Round 2], C [Round 3], and D [Round 4]). A PrP^{CWD} signal can be found in the negative '0' control by the fourth round of PMCA. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed) and 'L' molecular weight ladder (50kD, 37kD, and 25kD standards shown).

3.2.2.5 Reproducibility of the standardized PMCA PrP^{CWD} protocol and application to other cervid species

The optimized PMCA assay for PrP^{CWD} was shown to be consistently reproducible between independent experimental designs using a single source of cervid infectious brain homogenate (Fig 3.12). Moreover, the optimized PMCA assay was shown to be capable of ultra-sensitive detection of PrP^{CWD} in four different cervid species: white-tailed deer (Fig 3.13), elk (Fig. 3.14), mule deer (Fig 3.15), and red deer (Fig 3.16). The PrP^{CWD} signal could be consistently detected in all four different cervid species with at least 4-6 $\log_{10}$ sensitivity over standard Western blot of the original brain homogenates. After three rounds of amplification, the PrP^{CWD} signal could be detected in dilutions of a 20% infectious elk brain homogenate as low as 1: 10^{11}(Fig. 3.14).

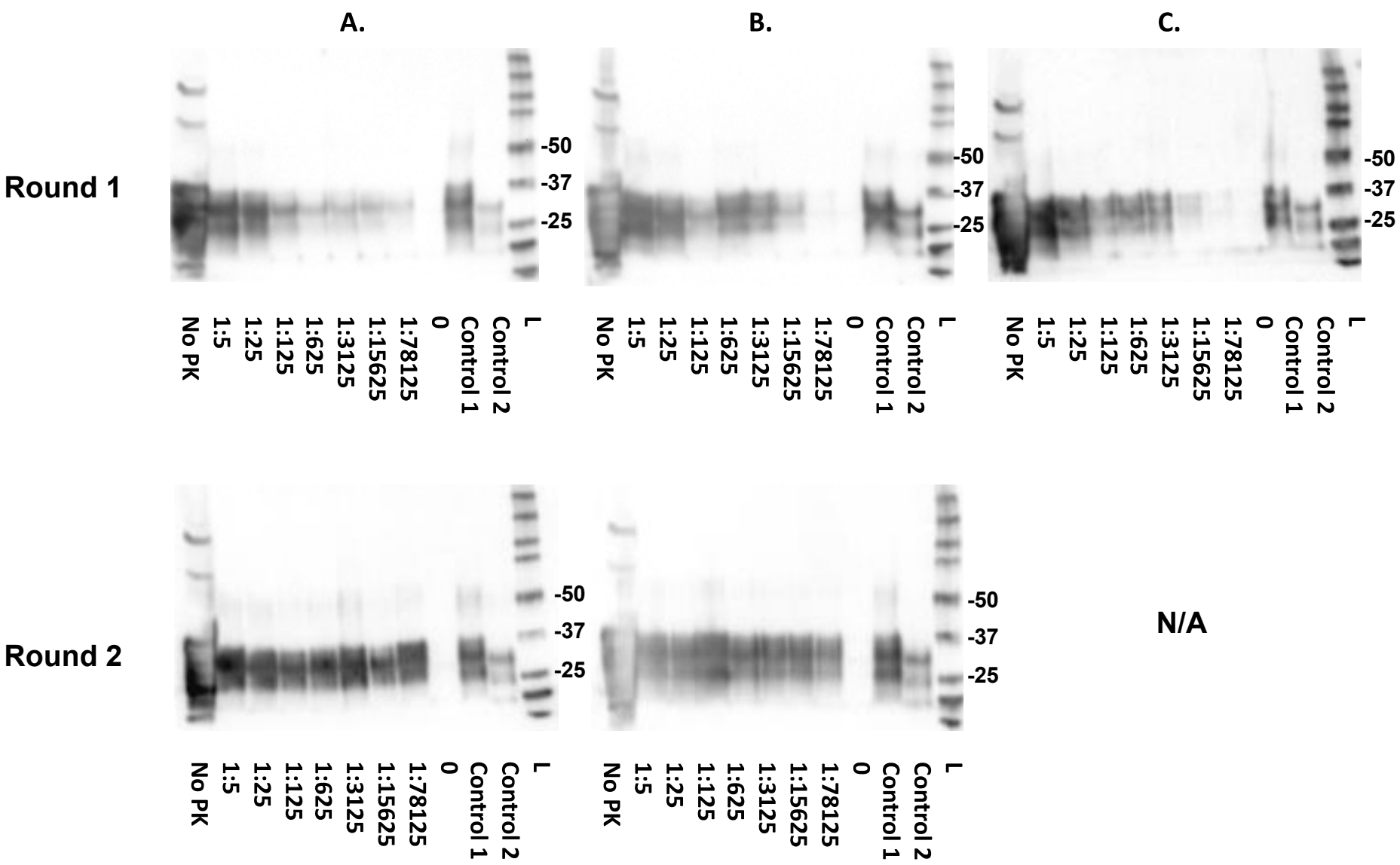
A.
B.
C.
Round 1
Round 2
N/A
-50
-37
-25
No PK
1:5
1:25
1:125
1:625
1:3125
1:15625
1:78125
0
Control 1
Control 2
L

FIGURE 3.12: WESTERN BLOTS DEMONSTRATING CONSISTENT IN VITRO AMPLIFICATION OF PRP^{CWD} USING THE STANDARDIZED PMCA PRP^{CWD} PROTOCOL:

95% power for 18 hours constituting 37 cycles, frozen at -80°C for 20 minutes, thawed at room temperature and treated with 250ug/mL Proteinase K diluted in 0.1% Triton X, 4% SDS in BioRad PBS for a final concentration of 50ug/mL for 30 minutes at 45°C. Replicates (Panels A, B and C) were from independent experiments where 20% elk infectious brain homogenate was serially diluted into 10% TgElk normal brain homogenate (labeled 1:5 – 1: 78125) and subjected to 2 rounds of PMCA (no second round for C was performed). Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed), 'Control 1' – 1:5 dilution of a 10% IBH sample stored at -80^{o}C and not subjected to PMCA, 'Control 2' – 1:5 dilution of a 10% elk IBH sample into conversion buffer, stored at -80^{o}C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

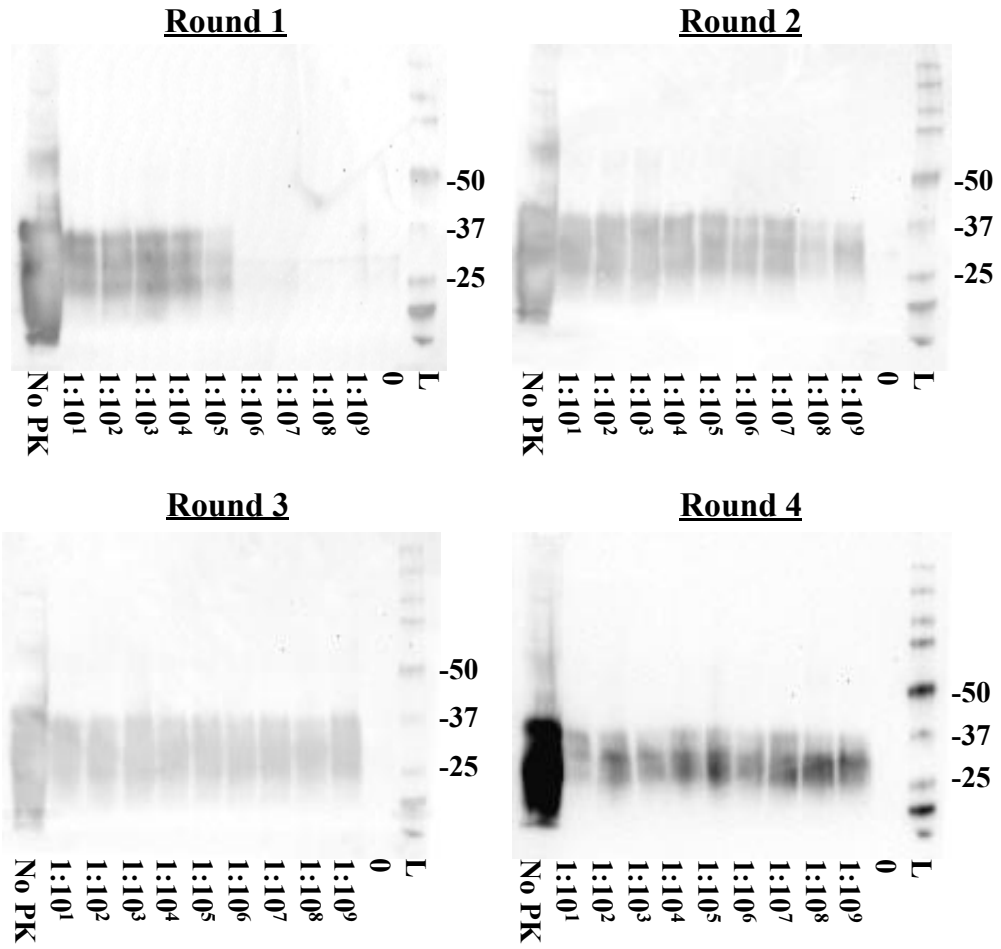

FIGURE 3.13: WESTERN BLOTS DEMONSTRATING PMCA AMPLIFICATION OF EXPERIMENTALLY INFECTED WHITE-TAILED DEER CWD.

A 20% white-tailed deer infectious brain homogenate (IBH) was serially diluted into 10% TgElk normal brain homogenate (labeled $1:10^1$ – $1:10^9$) and subjected to 4 rounds of PMCA (panels labeled Rd 1, Rd 2, Rd 3 and Rd 4). Before amplification, the PrP^{CWD} signal could not be detected in the IBH (not shown). After the first round of amplification, PrP^{CWD} could be detected in dilutions up to $1:10^5$. By the second round, PrP^{CWD} was detected up to the maximum dilution of $1:10^9$. All negative controls remained negative through 4 rounds of amplification. Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K, "0" – NBH only (no PrP^{CWD} seed) and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

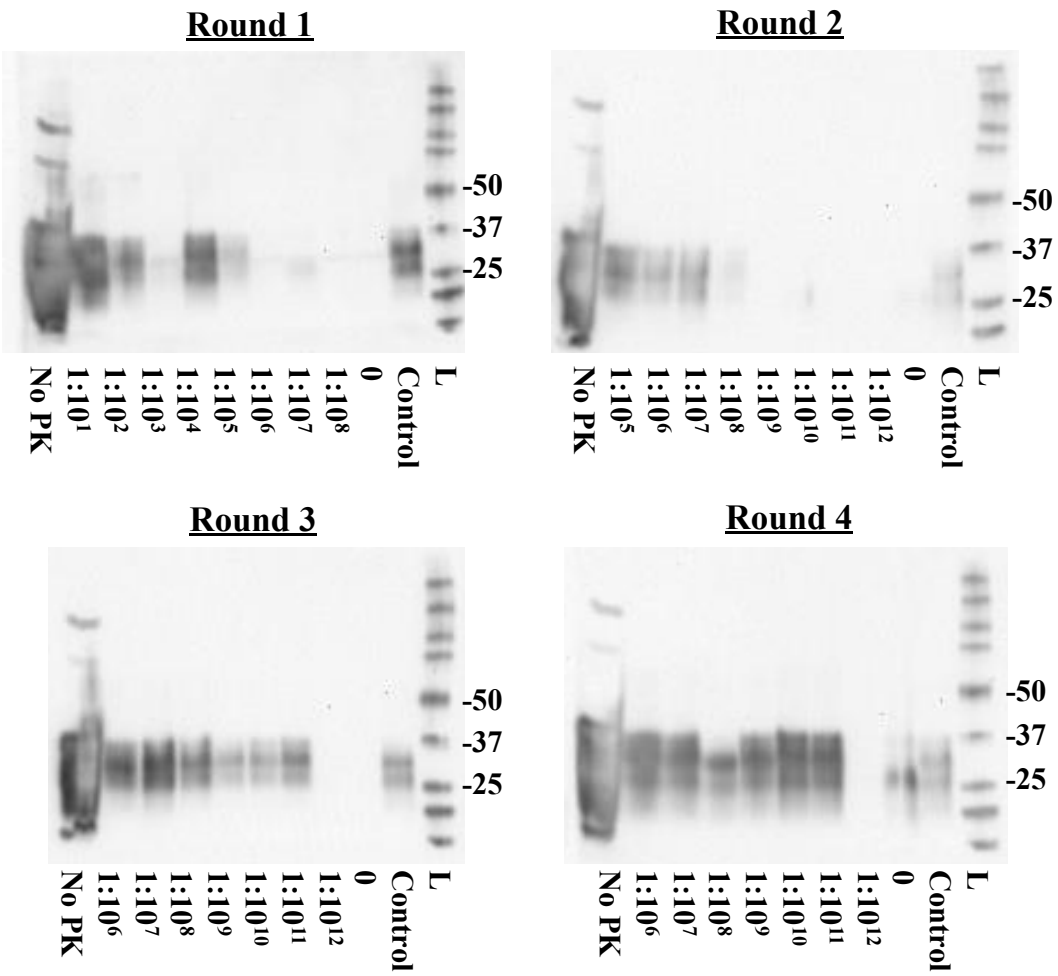

FIGURE 3.14: WESTERN BLOTS DEMONSTRATING PMCA AMPLIFICATION OF EXPERIMENTALLY INFECTED ELK CWD.

A 20% elk infectious brain homogenate (IBH) was serially diluted into 10% TgElk normal brain homogenate (labeled $1:10^1$ – $1:10^{12}$) and subjected to 4 rounds of PMCA (panels labeled Rd 1, Rd 2, Rd 3 and Rd 4). After the first round of amplification, PrP^{CWD} could be detected up to dilutions of $1:10^5$. By the third round, PrP^{CWD} was detected up to dilutions of $1:10^{11}$. All negative controls remained negative until round 4. Other lanes are designated as follows: "No PK" – NBH not treated with proteinase K, "0" – NBH only (no PrP^{CWD} seed), "Control 1" – 1:10 dilution of a 10% IBH sample stored at -80°C and not subjected to PMCA and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

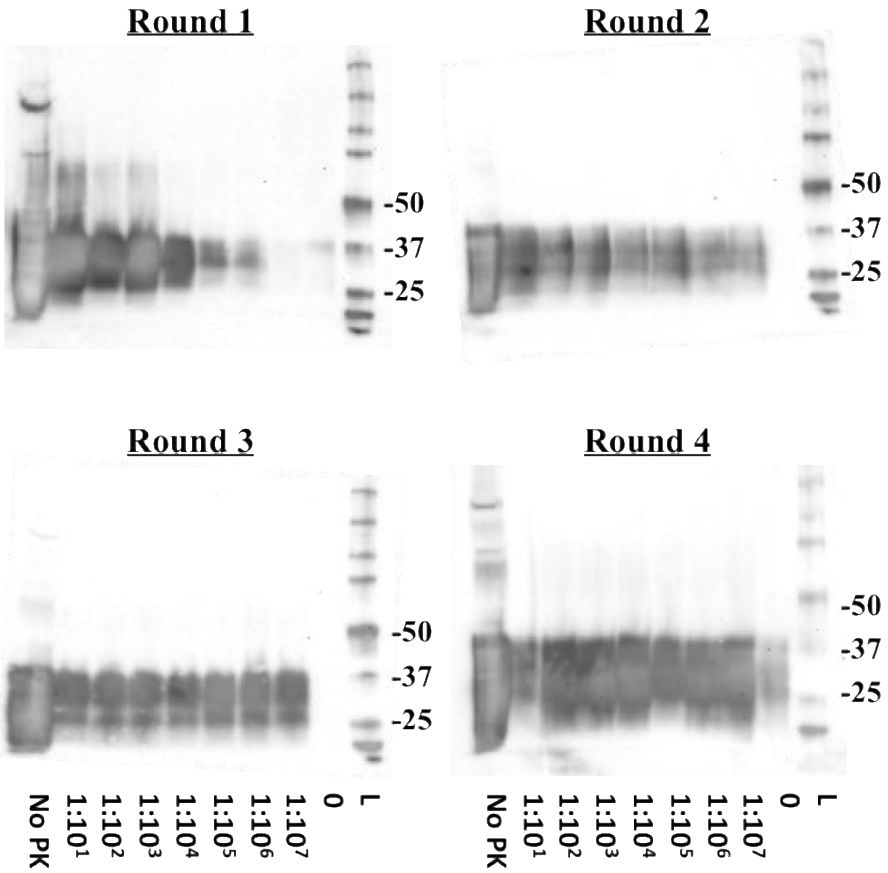

FIGURE 3.15: WESTERN BLOTS DEMONSTRATING PMCA AMPLIFICATION OF NATURALLY INFECTED MULE DEER CWD.

A 20% mule deer infectious brain homogenate (IBH) was serially diluted into 10% TgElk normal brain homogenate (labeled $1:10^1$ – $1:10^7$) and subjected to 4 rounds of PMCA (panels labeled Rd 1, Rd 2, Rd 3 and Rd 4). Before amplification, the PrP^{CWD} signal could not be detected in the infectious brain homogenate (not shown). After the first round of amplification, PrP^{CWD} could be detected in dilutions up to $1:10^6$. By the second round, PrP^{CWD} was detected up to the maximum dilution of $1:10^7$. All negative controls remained negative until round 4. Other lanes are designated as follows: "No PK" – NBH not treated with proteinase K, "0" – NBH only (no PrP^{CWD} seed) and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

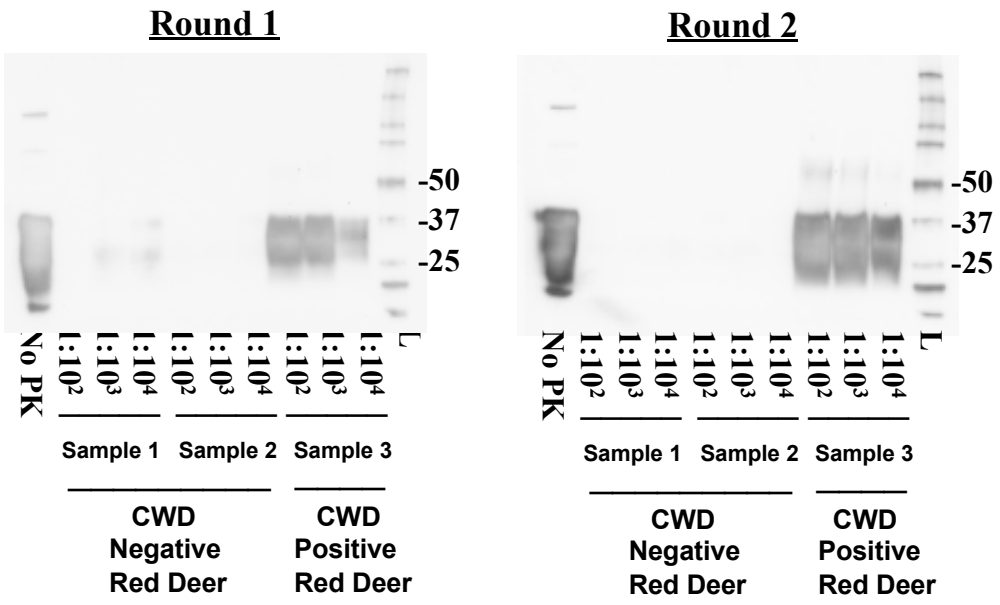

FIGURE 3.16: WESTERN BLOTS DEMONSTRATING PMCA AMPLIFICATION OF NATURALLY INFECTED RED DEER CWD.

20% red deer brain homogenates from individuals deemed positive or negative by conventional CWD tests (immunohistochemistry, ELISA and Western blot) were serially diluted into 10% TgElk normal brain homogenate (labeled $1:10^2$ – $1:10^4$) and subjected to 2 rounds of PMCA (panels labeled Rd 1 and Rd 2). Before amplification, the PrP^{CWD} signal could not be detected in any of the brain homogenates (not shown). After the first round of amplification, PrP^{CWD} could be detected in the sample deemed positive up to dilutions of $1:10^4$ while no signal was seen in either round of the negative samples. Other lanes are designated as follows: "No PK" – NBH not treated with proteinase K and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

3.2.3 Comparison of PMCA CWD protocols to other reports

Detection of PrP^{CWD} from the brain homogenate of a CWD-positive cervid was possible down to a dilution of 1:100,000, representing amplification of at least a 5 $\log_{10}$ increase in sensitivity from the initial level, in one 18-hour round consisting of 37 cycles of 40 seconds of sonication every half-hour using TgElk as

the NBH. Application of sPMCA resulted in some cases in an 11 $\log_{10}$ increase in sensitivity. By comparison, at this time other labs were also reporting amplification levels with 100% specificity. Haley *et al.* (2009b) reported that three rounds of amplification permitted an approximate 4000-fold increase in sensitivity using the Tg5037 transgenic mouse line and Nichols *et al.* (2009) reported the detection and amplification of a 1.3 x 10^{-7} dilution of CWD-infected brain homogenate into water, equivalent to approximately 5 x 10^{7} protease resistant cervid prion protein (PrP^{CWD}) monomers, also using the Tg5037 mouse line. By 2012, Johnson *et al.*, aided with saponin and Teflon beads, reported detection of the CWD agent from a 6.7 x 10^{-13} dilution of 10% brain homogenate (1.3 fg of source brain). All these other protocols required 144 hours to complete their steps. A summary of amplification comparisons and PMCA conditions is provided in Table 3.1.

Table 3.1: Comparison of PMCA CWD protocols

Name	Year	Detection limit	Time (Hrs)	Rounds	Cycles	Tg mouse NBH	Antibody
Price	2009	1.0*10e-5	18	1	37	TgElk	6H4
Nichols	2009	1.3*10e-7	144	6	48	Tg(CerPrP)5037	Bar 224
Haley	2009	4.0*10e-3	144	3	96	Tg5037	Bar 224
Johnson	2012	6.7*10e-13	144	3	96	Tg(CerPrP)1536	8G8

CHAPTER 4: APPLICATION OF PMCA FOR DETECTION OF CHRONIC WASTING DISEASE IN NATURALLY AND EXPERIMENTALLY-INFECTED WHITE-TAILED DEER[†]

4.1 Introduction

Animal bioassays are considered the gold standard for determining prion infectivity but the high cost, long incubation periods, and the maintenance of infected animals for several months to years (depending on the species), makes it extremely inconvenient and challenging for these assays to be used for general high-throughput diagnostic purposes. Currently, three types of diagnostic tests are routinely used to screen for CWD: enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC) and Western blot (WB). These diagnostic tests are dependent on detection of protease-resistant prions (PrP^{CWD}) in target tissues, such as the obex or lymph nodes (e.g., retropharyngeal or rectal lymph nodes). In typical CWD surveillance programs, ELISA is initially conducted on retropharyngeal lymph nodes and obex (if available), followed by IHC and Western blot on any samples with an ELISA optical density reading above established cutoff values. IHC provides information on sample integrity and the anatomical distribution of PrP^{CWD} while Western blot confirms the presence of protease-resistant prions in a banding pattern consistent with CWD.

Recently, the protein misfolding cyclic amplification (PMCA) assay has emerged as a potentially ultrasensitive *in vitro* test for detection of prions in

[†] A version of this Chapter has been submitted for publication: Price, L.M., S. L. Braithwaite, A. Balachandran, G. B. Mitchell, N.Yogasingam, M. Belosevic, and N. F. Neumann. 2013. Detection of chronic wasting disease in white-tailed deer by protein misfolding cyclic amplification (PMCA). PLoS One (submitted).

infected tissues. PMCA relies on the recruitment and template directed misfolding of normal prions (PrP^{C}) by an infectious prion seed (PrP^{Res}). Sequential cycles of incubation followed by sonication results in elongation of PrP^{Res} fibrils during the incubation phase and dispersion into smaller seeds during the sonication phase, resulting in an overall amplification of PrP^{Res} concentrations in vitro. If a PrP^{Res} seed is not present, little or no amplification occurs (Saborio *et al.*, 2001). In some cases, *de novo* production of PrP^{Res} has been observed after several rounds of PMCA. Applying multiple rounds of PMCA (also known as serial PMCA [sPMCA]), results in extremely sensitive detection of PrP^{Res} *in vitro*.

The PMCA method has been used to detect infectious prions in tissues such as tonsils, spleen, retina (Williams, 2005), skeletal muscle (Angers *et al.*, 2006), peripheral nervous system, endocrine organs, lymph nodes, spinal cord and brain (Williams, 2005) from many mammalian species, as well as environmental samples such as excrement (Haley *et al.*, 2009b), soil (Nagaoka *et al.*, 2010) and even water from endemic areas (Nichols *et al.*, 2009). Serial PMCA has been used to specifically amplify CWD prions in tissues of infected cervids (Kurt *et al.*, 2007). However, a comparative assessment of PMCA to traditional diagnostic tests has never been reported in cervids. This chapter examines the correlation of PMCA to traditional diagnostic methods (ELISA, IHC, and WB) for detecting PrP^{CWD} in experimentally and naturally infected (farmed) white-tailed deer (WTD).

4.2 Results and Discussion

4.2.1 Detection of PrP^{CWD} in WTD obex and retropharyngeal lymph node tissues by PMCA

PrP^{C} obtained from transgenic mice (TgElk) acted as an efficient template for amplification of PrP^{CWD} from WTD tissues (Fig. 4.1). Both obex and retropharyngeal lymph node (RLN) tissues obtained from CWD-infected animals were shown to be effective at seeding the PMCA assay (Fig. 4.1). Brain homogenates (20%) from infected animals could be diluted as low as 10^{-9} and the PrP^{CWD} could be detected in these tissues after two rounds of sPMCA (Fig. 4.1: Panel A). Similarly, PrP^{CWD} from RLN homogenates diluted as low as 10^{-5} could also be detected after 2 rounds of sPMCA. No PrP^{CWD} signal was detected after three rounds of PMCA in negative control samples (i.e., TgElk mouse brain samples only, data not shown).

4.2.2 Comparison of PMCA to traditional diagnostic tests for detection of CWD in WTD

Validation of the PMCA assay as a potential diagnostic tool was carried out on obex and RLN tissues from three WTD experimentally-infected with CWD. All animals progressed to clinical stages of disease and were euthanized 21-months post-inoculation (PI). All three deer were strongly positive for CWD based on ELISA (obex, RLN), IHC (obex, RLN, and tonsil), and Western blot (obex, RLN) (Fig. 4.2). Similarly, all three animals were shown to be positive for CWD by PMCA in the obex and RLN (Fig. 4.2). A single 18-hour round of PMCA was sufficient for detecting PrP^{CWD} in all three animals using either the obex or RLN tissues as a seed for PMCA (Fig. 4.2).

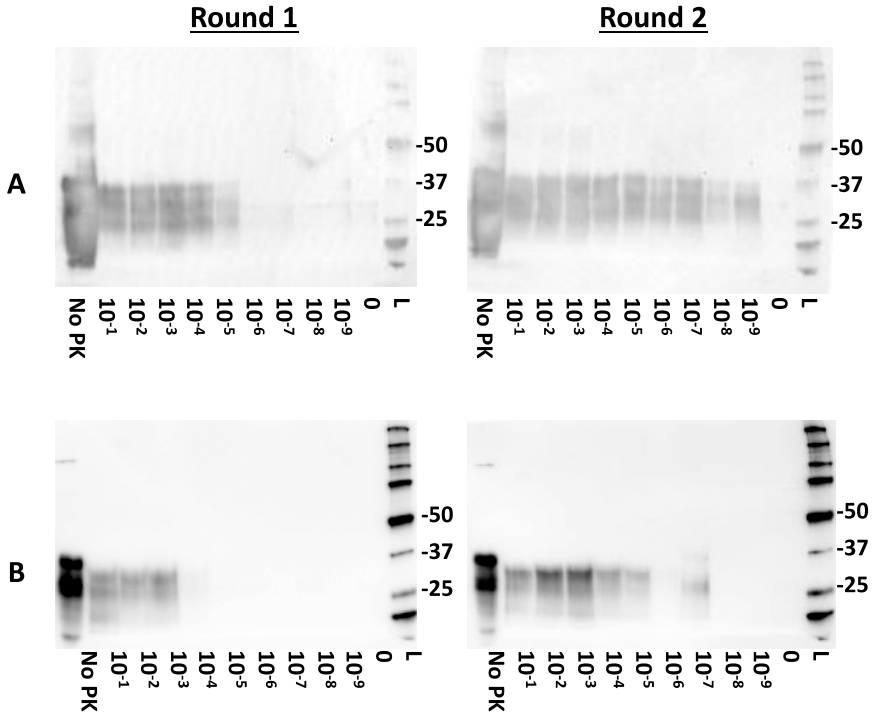

FIGURE 4.1: WESTERN BLOTS SHOWING DETECTION OF PRP^{CWD} IN THE OBEX AND RETROPHARYNGEAL LYMPH NODE TISSUES OF WHITE-TAILED DEER BY SERIAL PMCA.

Panel A: 20% WTD obex IBH serially diluted into 10% TgElk NBH (labeled 10^{-1} – 10^{-9}) and amplified by sPMCA. Before amplification, the PrP^{CWD} signal was only detectable in undiluted infectious brain homogenate (not shown). After the first round of amplification, PrP^{CWD} could be detected to the dilution of 10^{-5}, whereas PrP^{CWD} was detected in the second round at a 10^{-9} dilution of IBH. Panel B: 20% WTD retropharyngeal lymph node (RLN) homogenate serially diluted into 10% TgElk NBH and amplified by sPMCA. After the first round, PrP^{CWD} could be detected up to a 10^{-3} dilution of RLN homogenate and was detected in the second round at a dilution 10^{-5}. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed), 'Control 1' – 1:5 dilution of a 10% IBH sample stored at -80^{o}C and not subjected to PMCA, 'Control 2' – 1:5 dilution of a 10% elk IBH sample into conversion buffer, stored at -80^{o}C and not subjected to PMCA and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

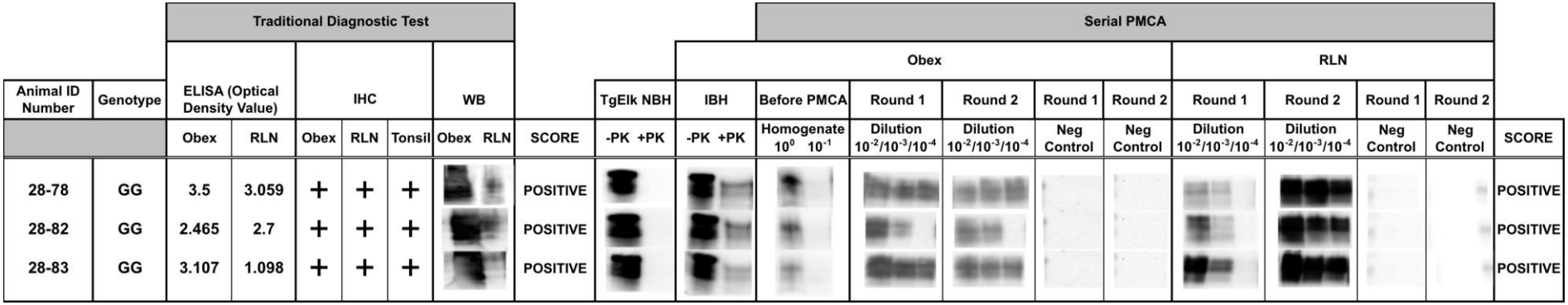

FIGURE 4.2: CORRELATION BETWEEN TRADITIONAL DIAGNOSTIC TESTS (ELISA, IHC AND WB) AND SERIAL PMCA FOR DETECTION OF CWD IN EXPERIMENTALLY-INFECTED WTD.

Obex and retropharyngeal lymph node (RLN) homogenate samples (20%) from experimentally-infected, and clinically-ill WTD deer were analyzed by traditional diagnostic methods (ELISA, IHC and WB) and sPMCA. Traditional diagnostic methods were performed at the National OIE Reference Laboratory for CWD in Nepean, Ontario (Dr. Balachandran). Two rounds of sPMCA were performed on obex and RLN homogenates at various dilutions (10^{-2} – 10^{-4}). Negative controls (Neg Control) represent samples subjected to PMCA but contain only TgElk NBH (and which act as controls for PK digestion of PrP^C in NBH). Unamplified homogenate samples with and without PK digestion (labeled 'IBH') are included for comparison to PMCA amplified samples in order to demonstrate amplification levels. Scoring of samples (i.e., positive or negative) based on traditional and PMCA results are also provided.

To assess the diagnostic credibility of sPMCA for detection of CWD, we performed comparative diagnostic trials on blinded tissue samples from WTD originating on CWD outbreak farms in Canada. Animals were screened by ELISA using the obex and RLN as diagnostic tissues. The designation of a 'negative' animal was based on an ELISA optical density (OD) value of < 0.201. In cases where ELISA OD values were > 0.04 secondary and tertiary confirmatory testing by IHC (obex, RLN and tonsil) and Western blot (obex and RLN) were performed. In the blinded panel of 29 animals, 17 deer were positive for CWD when employing the traditional diagnostic algorithms of ELISA (obex, RLN) followed by IHC (obex, RLN and tonsil) and Western blot (obex, RLN) on tissue samples (Fig. 4.3). All 17 CWD positive animals were also shown to be positive by sPMCA (Fig. 4.3). Examining individual test results on obex alone, 9/17 animals were classified positive by ELISA, 11/17 by IHC and 11/17 by Western blot (Fig. 4.3). By comparison, 17/17 CWD positive animals were deemed positive by sPMCA (two rounds) when the obex was used as the diagnostic tissue (Fig. 4.3). When the RLN was used as the diagnostic tissue, 16/17 animals were positive by ELISA, 17/17 by IHC, and 16/17 by Western blot (Fig. 4.3). Comparatively, all 17 animals were determined to be CWD positive by sPMCA using the RLN as the diagnostic tissue (Fig. 4.3).

Semi-quantitative estimates of CWD burden by PMCA also correlated well with ELISA values. For example, in tissue samples yielding high OD values by ELISA (i.e., > 1.0), often times only one round of PMCA was needed to detect the PrP^{CWD} signal in these tissues. Low ELISA values (e.g., ELISA on obex samples from animals 12-4, 12-7, 12-9, and 12-13; Fig. 4.3) often required 2 rounds of

PMCA in order to detect PrP^{CWD} in these samples. Interestingly, CWD positive animals homozygous for the SS genotype (resistant) appeared to have lower tissue (obex and RLN) burdens of CWD compared to GS heterozygotes (susceptible) and GG homozygotes (susceptible) as determined by ELISA and sPMCA (Fig. 4.3). In all cases, sPMCA could be used to amplify CWD from both resistant (SS) and susceptible genotypes (GG and GS) of infected farmed WTD (Fig. 4.3).

		Traditional Diagnostic Methods							Serial PMCA									
									Obex					RLN				
Animal ID Number	Genotype	ELISA (Optical Density Value)		IHC			WB		Before PMCA	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	
		Obex	RLN	Obex	RLN	Tonsil	Obex RLN	Score	Homogenate 10^0 10^{-1}	Dilution $10^{-2}/10^{-3}/10^{-4}$	Dilution $10^{-2}/10^{-3}/10^{-4}$	Neg Control	Neg Control	Dilution $10^{-2}/10^{-3}/10^{-4}$	Dilution $10^{-2}/10^{-3}/10^{-4}$	Neg Control	Neg Control	Score
12-3	GG	3.182	2.1	+	+	+		POSITIVE										POSITIVE
12-6	GG	3.5	3.285	+	+	+		POSITIVE										POSITIVE
12-12	GG	3.295	3.5	+	+	+		POSITIVE										POSITIVE
12-18	GG	3.408	3.5	+	+	+		POSITIVE										POSITIVE
12-20	GG	3.5	2.708	+	+	+		POSITIVE										POSITIVE
12-4	GS	0.074	2.257	-	+	+		POSITIVE										POSITIVE
12-5	GS	3.5	3.025	+	+	+		POSITIVE										POSITIVE
12-8	GS	0.033	3.294	+	+	+		POSITIVE										POSITIVE
12-10	GS	3.5	2.519	+	+	+		POSITIVE										POSITIVE
12-11	GS	0.447	2.472	+	+	+		POSITIVE										POSITIVE
12-15	GS	0.037	2.019	-	+	+		POSITIVE										POSITIVE
12-16	GS	0.03	0.345	-	+	+		POSITIVE										POSITIVE
12-17	GS	0.092	3.215	-	+	+		POSITIVE										POSITIVE
12-19	GS	3.5	2.647	+	+	+		POSITIVE										POSITIVE
12-7	SS	0.04	0.321	-	+	+		POSITIVE										POSITIVE
12-9	SS	0.052	1.484	-	+	+		POSITIVE										POSITIVE
12-13	SS	0.11	0.129	+	+	+		POSITIVE										POSITIVE

FIGURE 4.3: COMPARISON OF SERIAL PMCA FOR DETECTION OF CWD IN FARMED WTD SCORING POSITIVE FOR CWD BY THE TRADITIONAL DIAGNOSTIC TEST PANEL (ELISA, IHC AND WB).

Tissue samples (brain, RLN and tonsil) were analyzed by traditional methods and sPMCA (2 rounds). Sample homogenates for both obex and RLN were diluted from 10^{-2} -10^{-4} prior to conducting sPMCA. Traditional diagnostic methods were performed at the National OIE Reference Laboratory for CWD in Nepean, Ontario (Dr. Balachandran). Negative controls (Neg Control) represent samples subjected to PMCA but contain only TgElk NBH. Unamplified homogenate samples (Before PMCA) are included to demonstrate amplification levels.

Samples from CWD negative animals were also included in the blinded panel (Fig. 4.4). Of particular note, however, was that all negative animals (as determined by traditional diagnostic algorithms) originated from farms in which a CWD outbreak was confirmed. Twelve animals from CWD-infected farms were determined to be negative (one deemed as potentially suspect) by traditional diagnostic methods (Fig. 4.4). Interestingly, 3/12 animals appeared to be positive for CWD by sPMCA when the obex was used as the diagnostic tissue of choice (animals 12-14, 2-49 and 2-60), whereas 4/12 animals appeared positive by sPMCA when using the RLN as a diagnostic tissue (animals 12-14, 2-80, 2-49 and 2-60). The three animals in which the obex was positive by sPMCA (animals 12-14, 2-49, and 2-60, Fig. 4) were also positive in the RLN by sPMCA, suggesting that these animals were likely harboring CWD at pre-clinical/pre-diagnostic levels (i.e. traditional algorithms). The one ‘suspect’ case in the blinded panel (animal 12-14, Fig. 4) had an ELISA value below the OD cutoff value of 0.2 in obex (also negative by ELISA in the RLN), IHC positive by RLN

(but not in brain or tonsil) and negative in both obex and RLN by Western blot. sPMCA confirmed this animal as being positive for CWD based on detection of PrP^{CWD} in both the obex and RLN.

Animal ID Number	Genotype	Traditional Diagnostic Methods							Serial PMCA								
		ELISA (Optical Density Value)		IHC			WB		Obex				RLN				
									Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	Round 1	Round 2	
		Obex	RLN	Obex	RLN	Tonsil	Obex RLN	Score	Dilution $10^{-2}/10^{-3}/10^{-4}$	Dilution $10^{-2}/10^{-3}/10^{-4}$	Neg Control	Neg Control	Dilution $10^{-2}/10^{-3}/10^{-4}$	Dilution $10^{-2}/10^{-3}/10^{-4}$	Neg Control	Neg Control	Score
12-1	GS	0.03	0.156	-	-	-		NEGATIVE					NA	NA	NA	NA	NEGATIVE
12-2	GS	0.028	0.028	N/A	N/A	N/A		NEGATIVE									NEGATIVE
12-14	SS	0.049	0.036	-	+	-		SUSPECT									POSITIVE
2-91	No DNA	0.009	0.012	N/A	N/A	N/A	N/A	NEGATIVE									NEGATIVE
2-78	No DNA	0.011	0.013	N/A	N/A	N/A	N/A	NEGATIVE									NEGATIVE
2-80	No DNA	0.006	0.013	N/A	N/A	N/A	N/A	NEGATIVE									POSITIVE
2-71	No DNA	0.008	0.013	N/A	N/A	N/A	N/A	NEGATIVE									NEGATIVE
2-72	No DNA	0.008	0.01	N/A	N/A	N/A	N/A	NEGATIVE									NEGATIVE
2-105	No DNA	0.012	0.01	N/A	N/A	N/A	N/A	NEGATIVE									NEGATIVE
2-49	No DNA	0.01	0.013	N/A	N/A	N/A	N/A	NEGATIVE									POSITIVE
2-81	No DNA	0.012	0.014	N/A	N/A	N/A	N/A	NEGATIVE									NEGATIVE
2-60	No DNA	0.011	0.009	N/A	N/A	N/A	N/A	NEGATIVE									POSITIVE

FIGURE 4.4: COMPARISON OF SERIAL PMCA FOR DETECTION OF CWD IN FARMED WTD SCORING NEGATIVE (OR SUSPECT) FOR CWD BY THE TRADITIONAL DIAGNOSTIC TEST PANEL (ELISA, IHC AND WB).

Tissue samples (brain, RLN and tonsil) were analyzed by traditional methods and sPMCA (2 rounds). Twenty percent sample homogenates for both obex and RLN were diluted from 10^{-2} -10^{-4} prior to conducting sPMCA. Negative controls (Neg Control) represent samples subjected to PMCA but contain only TgElk NBH.

A blind study was also performed on obex samples collected from a hunter harvest of mule deer near Wainwright, Alberta in the fall of 2011 (Fig. 4.5). The PrP^{CWD} signal was only detectable in samples identified as positive by Alberta Agriculture's TSE unit (#3, 4, 8, 9 and 12). No signal was observed in any of the negative samples. A parallel set of unsonicated samples was included to demonstrate the necessity of amplification for detection.

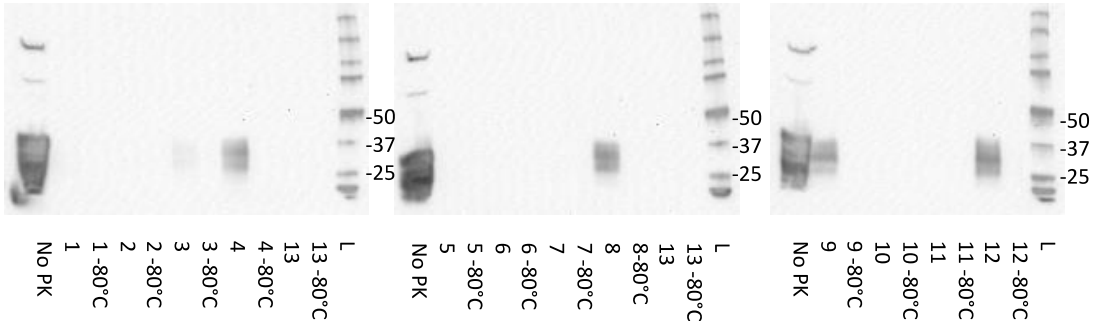

FIGURE 4.5: WESTERN BLOTS SHOWING DETECTION OF PRP^{CWD} IN BLINDED OBEX SAMPLES FROM NATURALLY INFECTED WILD MULE DEER BY SERIAL PMCA.

A 1:10 dilution of 10% mule deer obex homogenate into 10% TgElk NBH was performed for 13 blinded samples and amplified by sPMCA. The PrP^{CWD} signal was detected in samples #3, 4, 8, 9 and 12. Sample numbers designated with -80°C indicate no sonication for that sample occurred. Other lanes are designated as follows: 'No PK' – NBH not treated with Proteinase K, '0' – NBH only (no PrP^{CWD} seed) and 'L' – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

Ultra-sensitive detection of prions by sPMCA has been described in several model systems including CWD (Kurt *et al.*, 2007; Meyerett *et al.*, 2008), scrapie (Castilla *et al.*, 2005) and BSE (Soto *et al.*, 2005). As few as 26 molecules of hamster 263K PrP^{Sc}, equivalent to approximately one infectious particle, have

been detected using sPMCA (Saá *et al.*, 2006). Recent studies have demonstrated that the detection limit of PMCA exceeds animal bioassays by 4000 fold (Saá *et al.*, 2006). Similarly, the quaking-induced conversion assay (QUIC) has been shown to detect as little as 10^{-15} g of PrP^{Sc} in infected tissues (Atarashi *et al.*, 2008). Threshold detection levels for the Western blot and ELISA have been calculated at 4ng of PrP corresponding to $8x10^{10}$ PrP molecules and 0.5ng of PrP corresponding to $1x10^{10}$ PrP molecules respectively, while 2 rounds of sPMCA have been shown to detect 0.7fg of PrP^{Sc} or $1.3x10^{4}PrP^{Sc}$ molecules, almost a six million times increase in sensitivity (Saá *et al.*, 2006).

A definitive comparative study between the PMCA assay and current conventional tests for diagnosis of CWD (ELISA, IHC, and WB) has not been fully explored. Herein we demonstrate robust correlations between the conventional diagnostic test panel and PMCA. While all three conventional diagnostic tests (ELISA, IHC and Western blot) are considered reliable and widely used, the sensitivities of these individual assays are limited in some cases, requiring a combination of the different assays across multiple tissue types (obex, RLN and tonsil) to yield a confirmatory result for CWD. In the present study the conventional test panel (ELISA, IHC and Western blot) identified 17/29 animals as being PrP^{CWD} positive in a subset of WTD tested from farms implicated in outbreaks of CWD. Our results using obex and RLN as tissues for conventional diagnostic testing purposes are similar to those of others (Spraker *et al.*, 2002; Gavier-Widén *et al.*, 2005). Conversely, PMCA appears to achieve a high level of sensitivity for CWD when using either the obex or RLN as the diagnostic tissue of choice when compared to the conventional test panel results, suggesting that

PMCA may be an ultra-sensitive diagnostic test for CWD when compared to conventional diagnostic algorithms.

The high sensitivity of PMCA may also explain the enhanced detection of PrP^{CWD} in animals that were CWD-negative by conventional tests. Twelve animals were identified as being negative (with one suspect) for CWD based on the conventional test algorithms of ELISA, IHC, and Western blot on various tissues (obex, RLN and tonsil). However, of these 12 animals, sPMCA identified 4 as potentially being infected with CWD when the obex and RLN were used as the diagnostic tissues. Although it could be interpreted that PMCA may be less specific than the combined conventional test panel (i.e., higher false positive rate), it could be argued that due to the ultra-sensitive nature of the PMCA assay these animals may represent true positives, and therefore, by association, the conventional test panel may result in more 'false negatives' in this comparative study. Evidence to support the claim that PMCA may be more sensitive than conventional tests is based on several observations. Firstly, all CWD-negative animals, as determined by the conventional diagnostic test panel, originated from CWD-infected farms. Therefore, the prion agent responsible for disease transmission was present in the local environment of these so-called 'negative' animals. Secondly, from the 12 animals that were negative by conventional testing, three were CWD positive in both the obex and RLN by sPMCA, independently confirming the presence of the PrP^{CWD} in two different tissues within the same animal and across three individual animals. Thirdly, by conventional testing, RLN tissues have been shown to be more sensitive than obex (as described in this study and by others (Sigurdson *et al.*, 1999; Fox *et al.*,

2006), and therefore the observation that four animals were shown to be positive by sPMCA in RLN but only three were positive by obex, is not unexpected. It is widely accepted that PrP^{CWD} accumulation occurs in peripheral tissues before reaching the brain and therefore conventional diagnosis requires multiple tissues to be tested in order to produce a reliable result (Williams, 2005). Thus, the animals deemed negative by conventional diagnostic tests may have been harboring CWD infections at extremely low levels that were only detectable by PMCA, with one of the animals at an early stage of infection (RLN positive and obex negative). Fourthly, CWD prions were not detected by sPMCA in either the RLN or obex in eight animals and these same animals were also negative by conventional tests (even though all animals originated from infected farms). This suggests a 67% concurrent detection rate between the conventional methods and sPMCA for truly negative animals across multiple tests within an exposed population. Fifthly, one animal that was classified as 'suspect' by conventional diagnostic testing was subsequently confirmed to be highly positive in both the RLN and the obex by sPMCA. Together, the evidence suggests that PMCA may be an ultra-sensitive diagnostic test for CWD detection in WTD (and possibly in other cervids).

In order to conclusively resolve the disparity between conventional tests and PMCA, we are currently using animal infectivity bioassays (TgElk mice) to confirm the level of prion infectivity in RLN and obex samples from animals that were negative by conventional assays but positive by sPMCA. This will help determine whether pre-diagnostic WTD, based on conventional tests, were actually harboring CWD in early stages of disease at PrP^{CWD} levels below the

detection threshold for conventional methods. However, it has been demonstrated that the sensitivity of rodent bioassay studies may be insufficient to detect the lower levels of infectious prions circulating near the beginning of infection (Grassi *et al.*, 2008). Therefore, studies in transgenic mice may not accurately reflect whether the extremely low levels of infectious PrP^{CWD} detected by sPMCA are sufficient to cause CWD in longer-lived cervids. To address this it will be important to determine whether CWD-exposed WTD that screen negative for PrP^{CWD} in RLN by conventional tests but positive by sPMCA will eventually develop the disease after being removed from infected pasture and quarantined.

An interesting observation, representing a novel finding in this study, was the ability of PMCA to detect CWD in infected animals of different *Prnp* genotypes. A glycine/serine polymorphism at codon 96 of the *Prnp* gene in WTD appears to confirm some level of resistance to CWD in this species. White-tailed deer that are *S/S* homozygous are more resistant to CWD infection compared to *G/S* heterozygotes and *G/G* homozygotes (O'Rourke *et al.*, 1999). Interestingly, genotype resistance to CWD appeared to be reflected in the diagnostic outcomes of this study. White-tailed deer that were *S/S* homozygous at codon 96 had lower ELISA OD values (obex and RLN) than *G/G* genotypes, an observation which also correlated with PMCA. In all cases, two rounds of PMCA were needed to amplify PrP^{CWD} from *S/S* genotypes whereas only one round was required to amplify PrP^{CWD} from *G/G* genotypes. For G/S heterozygotes, ELISA OD values were intermediate, and either one or two rounds of PMCA were required to amplify PrP^{CWD} in these animals. In all cases, the outcomes of sPMCA were shown to be dose dependent. The 'semi-quantitative' nature of the PMCA assay

may be useful at estimating body burdens of PrP^{CWD} in tissues, secreta and excreta of infected animals, information that will be important in assessing characteristics of contagion, such as determining the dose of CWD associated with natural exposure scenarios (e.g., grooming behavior, environmental contamination levels in over wintering areas, etc.).

CHAPTER 5: Assessing PrP^{CWD} tissue tropism and shedding of CWD prions in the secreta/excreta of infected cervids using PMCA

5.1 Introduction

Although believed to be transmitted horizontally, the factors affecting transmission and contagion of chronic wasting disease among infected animals are largely unknown. The increased prevalence of the disease in endemic areas can greatly affect tranmission dynamics (e.g., dose, frequency of exposure, etc). The presence of infectious prions in bodily tissues and fluids suggests that secreta/excreta may be possible routes of transmission of CWD for cervid species. It has been shown through an oral inoculation route, that concentrated saliva samples and blood from infected deer are able to transmit CWD to naïve white-tailed deer (WTD) and produce incubation periods consistent with those observed in naturally acquired infections (Mathiason *et al.*, 2006). Prion infectivity has been detected by bioassay of concentrated, dialyzed urine and saliva in transgenic mice expressing the cervid PrP (Haley *et al.* 2009a). It has been recently shown that mule deer infected with CWD excrete infectious prions in their feces long before the onset of neurological symptoms, suggesting a possible long-term source for the horizontal spread (Tamguney *et al.*, 2009). Safar *et al.* (2008) have also shown that uninoculated Syrian hamsters (SHas) cohabitated with or exposed to the bedding of SHas orally infected with Sc237 prions have a rate of prion infection of 80%-100% with incubation times of ~140 days suggesting oral transmission via coprophagy (Safar *et al.*, 2008).

As outlined in Chapter 4, PrP^{CWD} can be detected in both neurological (obex) and lymphoid tissues (retropharyngeal lymph node) using PMCA. Critical knowledge gaps exist regarding tissue tropism of PrP^{CWD} in cervids under natural exposures: i.e., which tissues accumulate PrP^{CWD} and how soon after initial exposure does PrP^{CWD} deposition in these tissues occur based on exposure doses under natural conditions.

Under experimental oral inoculation of mule deer, early accumulation (40-45 days p.i.) of PrP^{CWD} has been detected in Peyer's patches, ileocecal lymph nodes and the retropharyngeal lymph nodes (Sigurdson *et al.*, 1999). Aproximately 90 days p.i., PrP^{CWD} deposits appear in tonsils and lymph nodes. By nine months p.i., PrP^{CWD} accumulation has been observed in the gut-associated lymphoid tissue (GALT) and ganglia of the enteric nervous system, as well as the dorsal motor nucleus of the vagus nerve and by 16 months PrP^{CWD} deposits have been detected throughout the brain and spinal cord (Sigurdson, 2008).

In addition, the burden of infection found within many tissues in WTD is largely unknown, particularly for those tissues that may be used for consumption purposes (i.e., muscle, spleen, kidney, liver, etc). In order to address these issues, white-tailed deer (WTD) were infected with PrP^{CWD} euthanized at various times post inoculation (PI), and assorted tissues were collected at necropsy. PrP^{CWD} was detected and burden of PrP^{CWD} semi-quantitated in tissues using PMCA. Secreta and excreta samples were also collected at specific time points and tested for the presence of PrP^{CWD} to assess time course of shedding of prions in WTD.

5.2 Results and Discussion

5.2.1 Tissue tropism of PrP^{CWD} in experimentally-infected WTD

White-tailed deer experimentally-infected with CWD were euthanized at specific time points during the course of infection and the presence of Pr^{CWD} assessed in spleen, heart, salivary gland, kidney, liver, skeletal muscle, adrenal glands and colon using PMCA (Table 5.1).

PrP^{CWD} was detected in the spleen, adrenal gland and colon as early as 2.5 months post inoculation (PI). In all cases, 2 rounds of PMCA were required to detect these apparently low concentrations of PrP^{CWD} in these tissues at this time point. Although a full quantitative analysis of PrP^{CWD} was not performed on all tissues, the spleen appeared to become more heavily infected than the adrenal gland early after infection. This conclusion was based on the finding that the PMCA assay could be used to detect PrP^{CWD} after only one round in spleen samples and across two dilutions (10^{-2} and 10^{-3}), compared to adrenal gland samples where only the lowest dilution (10^{-2}) resulted in detection of PrP^{CWD} and only in animals infected with CWD after 16 months. Levels of PrP^{CWD} appeared to persist in spleen samples throughout the course of the infection (up to 22 months). Interestingly, inhibition of PMCA was observed in colon tissue homogenate concentrations diluted 10^{-2}, but not at 10^{-3}. This finding was similar to what is reported for detection of PrP^{CWD} in feces (see below), suggesting that components of certain tissues may inhibit PMCA reactions. Nevertheless, PrP^{CWD} was detected as early as 2.5 months after infection and persisted in the colon of animals throughout the course of infection.

Table 5.1: Patterns of PrP^{CWD} accumulation in peripheral tissues of orally inoculated WTD sacrificed at post inoculation (PI) intervals

		=POSITIVE		=NEGATIVE		=NO SAMPLE									
		ORGAN													
		SPLEEN				HEART				SALIVARY GLAND				KIDNEY			
		PMCA (Round)															
Months	*Prnp*	Rd 1		Rd 2		Rd 1		Rd 2		Rd 1		Rd 2		Rd 1		Rd 2	
post	Codon	DILUTION OF ORGAN HOMOGENATE TESTED															
inoc.	96	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
2.5	N/A	-	-	+	-												
4	N/A	+	+	+	+	-	-	-	-								
11	GG	-	-	+	+	-	-	-	-					+	-	+	+
11	GS	+	+	+	+	-	-	-	-					-	-	+	-
16	GG	+	+	+	+	+	-	+	+					+	-	+	+
16	GG	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+
20	GG	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
20	GG	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
22	GG	+	+	+	+	+	-	+	+	+	-	+	+	-	-	+	+

Months	*Prnp*	LIVER				MUSCLE				ADRENAL GLAND				COLON			
post	Codon	Rd 1		Rd 2		Rd 1		Rd 2		Rd 1		Rd 2		Rd 1		Rd 2	
inoc.	96	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
2.5	N/A									-	-	+	-	-	-	-	+
4	N/A									-	-	+	+	-	-	+	+
11	GG	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+
11	GS	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+
16	GG	-	-	+	+	-	-	+	-	+	+	+	+	-	+	+	+
16	GG	-	-	+	+	-	-	+	+	+	-	+	+	-	+	+	+
20	GG	-	-	+	+	-	-	-	-	+	-	+	+	-	+	+	+
20	GG	-	-	+	+	-	-	-	-	+	-	+	+	-	+	+	+
22	GG	-	-	+	+	-	-	-	-	+	-	+	+	+	+	+	+

Almost no PrP^{CWD} was found in the striated muscle tissues during the course of infection, even at the later stages of disease, with the exception of samples collected from two animals at 16 months PI. In both cases, two rounds of PMCA were needed for detection of PrP^{CWD} in these animals suggesting relatively low levels of PrP^{CWD} in skeletal muscles. No PrP^{CWD} deposition was observed in skeletal striated muscles in 3 animals at later time points (i.e., 20 and 22 months). Arguably, biopsy material from striated muscle may contain varying amounts of nervous tissue that consequently leads to variable levels of PrP^{CWD} within these samples. Alternatively, recent research has also demonstrated an inhibitory effect of hemoglobin on PMCA (Castilla *et al.*, 2006; Tattum *et al.*, 2010), suggesting that tissues such as muscles that contain large amounts of hemoglobin may reduce detection sensitivity of PrP^{CWD} in these tissues by PMCA. However PrP^{CWD} was readily detected in cardiac muscle, a tissue also known to contain high levels of hemoglobin. PrP^{CWD} was detected in cardiac muscle as early as 16 months PI, and was present in cardiac muscle from all animals sampled after this time point (Table 5.1).

PrP^{CWD} was found consistently in salivary glands from all animals infected for $\geq$16 months. Unfortunately, salivary gland samples from animals euthanized at earlier time points were not available for analysis. PrP^{CWD} could be detected in salivary gland homogenates diluted 1:1000 after only a single round of PMCA, suggesting a relatively abundant amount of PrP^{CWD} within this tissue of infected animals.

Consistent, but low levels of PrP^{CWD} were found in the kidney and liver from 11 to 22 months PI. Concentrations of PrP^{CWD} appeared to be low in the liver, since two rounds of PMCA were needed to detect liver tissue deposits of PrP^{CWD} in all cases. For kidney homogenates, PrP^{CWD} could be detected after only a single round of PMCA and at the lowest dilution of homogenates (i.e., 10^{-2}) in some of the samples, but was consistently detected after second round PMCA.

Differences in PrP^{CWD} tissue deposition between two animals of the GG and GS genotypes at *Prnp* codon 96 and euthanized 11 months PI were also noted, with the GS animal having lower PrP^{CWD} levels in the kidney than the GG animal, and undetectable in the liver of the GS animal compared to the GG animal. Although interesting to speculate that genotype may also affect tissue tropism, the limited number of animals represented by the different genotypes in this study precludes drawing conclusions about *Prnp* codon 96 and the course of tissue tropism in WTD in these specific samples.

Collectively, these data suggest that little infectivity may be present in striated muscle tissues, while the burden of PrP^{CWD} may be significantly higher in many of the internal organs early after infection (i.e., pre-clinical), and remains high throughout the course of the disease in WTD. This raises concerns regarding the consumption of these internal organs by hunters or other demographic groups (i.e., First Nations people) who treat these organs as delicacies, since the exposure dose in this sub-population of individuals may be considerably different than those who consume only skeletal muscles of WTD.

5.2.2 Shedding of PrP^{CWD} in the secreta and excreta of CWD-infected WTD

Sensitivity limitations and time demands of conventional assays prevent the timely detection of prion loads in the secreta, excreta of animals as well as in the environment. This portion of the thesis focused on using sPMCA to detect PrP^{CWD} in WTD feces, urine and saliva through both spiking studies and from WTD experimentally infected with CWD. For method development studies, fecal homogenates prepared from CWD-negative WTD fecal pellets (animals originally assayed by traditional diagnostic algorithms), as well as urine and saliva were spiked with WTD-infectious brain homogenate and amplified by sPMCA.

The PMCA assay was subsequently used to detect PrP^{CWD} in the feces, urine and saliva of experimentally infected WTD for validation purposes. Pooled feces were collected from the pens of infected animals at four different time points (5, 10, 16 and 21 months post inoculation), urine samples were collected at 2, 4 and 21 months PI and saliva samples were collected at 21 months PI in CWD infected WTD.

5.2.2.1 Feces

Feces were shown to inhibit the sPMCA reaction when relatively high concentrations of feces were spiked within the PMCA reaction [1-10% w/v, Fig. 5.1]. This inhibitory effect could be overcome by simple dilution of the sample matrix in conversion buffer and did not compromise sensitivity of the sPMCA assay (Fig. 5.1). Three rounds of a fecal dilution series run in parallel, with and without the addition of IBH, were run to demonstrate that the PrP^{CWD} signal was

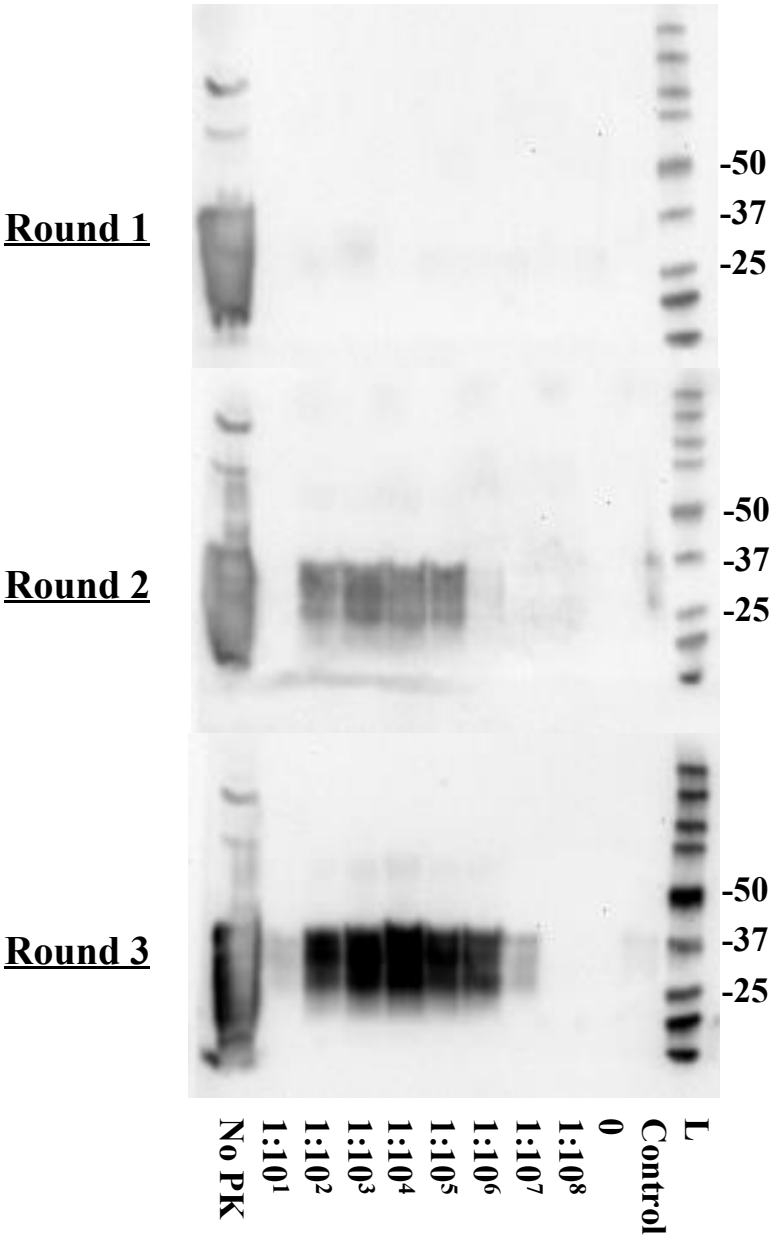

FIGURE 5.1: WESTERN BLOTS DEMONSTRATING AMPLIFICATION OF PrP^{CWD} FROM FECES USING sPMCA.

A 10% fecal homogenate sample spiked with 1% WTD IBH was serially diluted into 10% TgElk NBH (10^1 – 10^8). Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K, "0" – NBH only (no PrP^{CWD} seed), "Control" – dilution of a 10% IBH sample stored at -80ºC and not subjected to PMCA and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

not a result of cross contamination of sample or *de novo* replication facilitated by the presence of feces (Fig. 5.2). After three rounds of sPMCA sonication, the PrP^{CWD} signal was only detected in the dilution series that had been spiked with WTD IBH (compare Figs. 5.1 and 5.2).

After the first two rounds of sPMCA, PrP^{CWD} could be detected in the samples with diluted WTD IBH up to $1:10^5$ but was absent from the starting dilution of $1:10^1$ suggesting that the PMCA assay was inhibited by concentrations of feces $\geq$1% in the buffer. The effects of inhibition of PMCA were observed in first and second rounds of PMCA at a $1:10^1$ dilution. By the third round PrP^{CWD} banding was observed in the $1:10^1$ dilution and extended as far as the $1:10^7$ dilution. No PrP^{CWD} bands were observed in the parallel dilution series without the addition of WTD IBH (Fig. 5.2, lane labeled '0').

In order to better assess the inhibitory effect of feces on PrP^{CWD} detection by PMCA, a fecal preparation was spiked with $PrP^{CWD/Elk}$ (due to the ability to detect the elk CWD by standard Western Blot at a 1:10 dilution) and prepared as a lower dilution series (1:5 – 1:78,125). After three rounds of PMCA, $PrP^{CWD/Elk}$ could be detected in samples diluted 1:625 to 1:78,125 but not in the dilutions of 1:5 and 1:25 (Fig. 5.3). A reduced intensity of PrP^{CWD}- specific banding was visually observed in the 1:125 dilution.

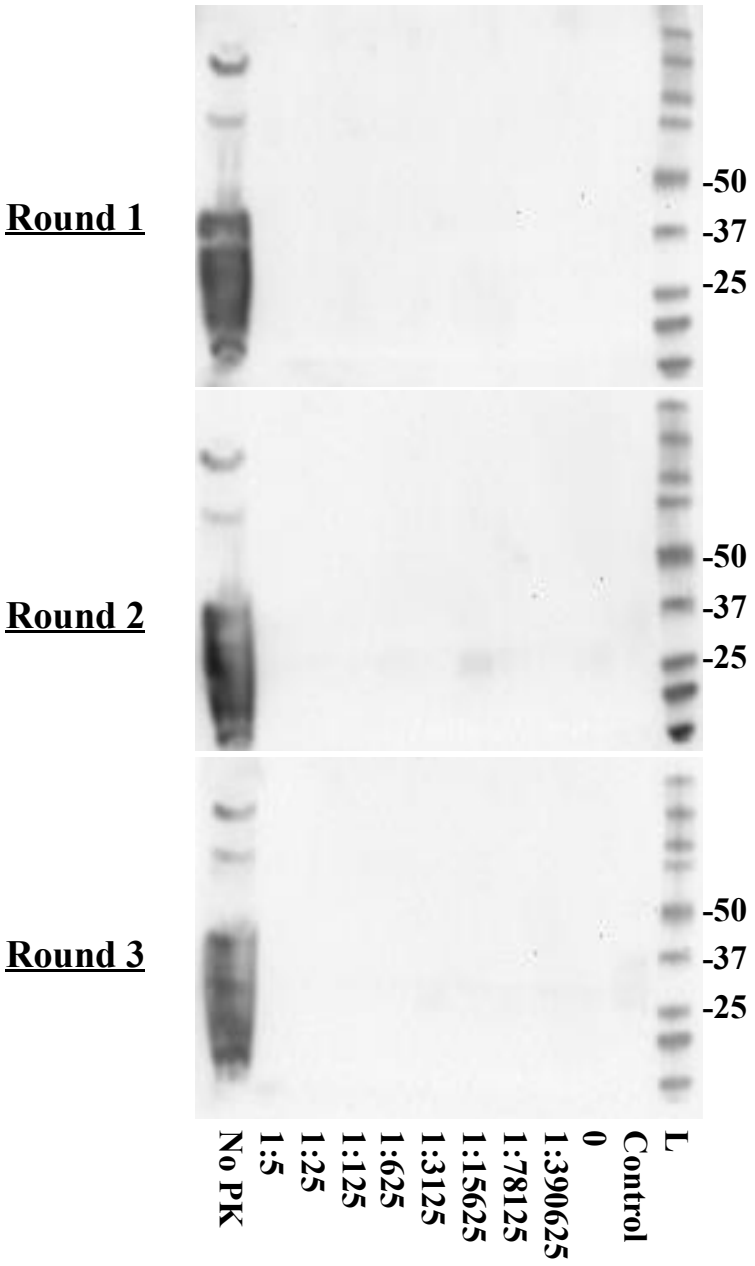

FIGURE 5.2: WESTERN BLOTS DEMONSTRATING THAT NO PrP^{CWD} SIGNAL IS PRODUCED FROM FECAL HOMOGENATE SUBJECTED TO PMCA BUT NOT SPIKED WITH PrP^{CWD}.

A 10% fecal homogenate sample from a CWD negative deer was serially diluted into 10% TgElk NBH and amplified. Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K, "0" – NBH only (no PrP^{CWD} seed), "Control" – dilution of a 10% WTD IBH sample stored at -80ºC and not subjected to PMCA and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

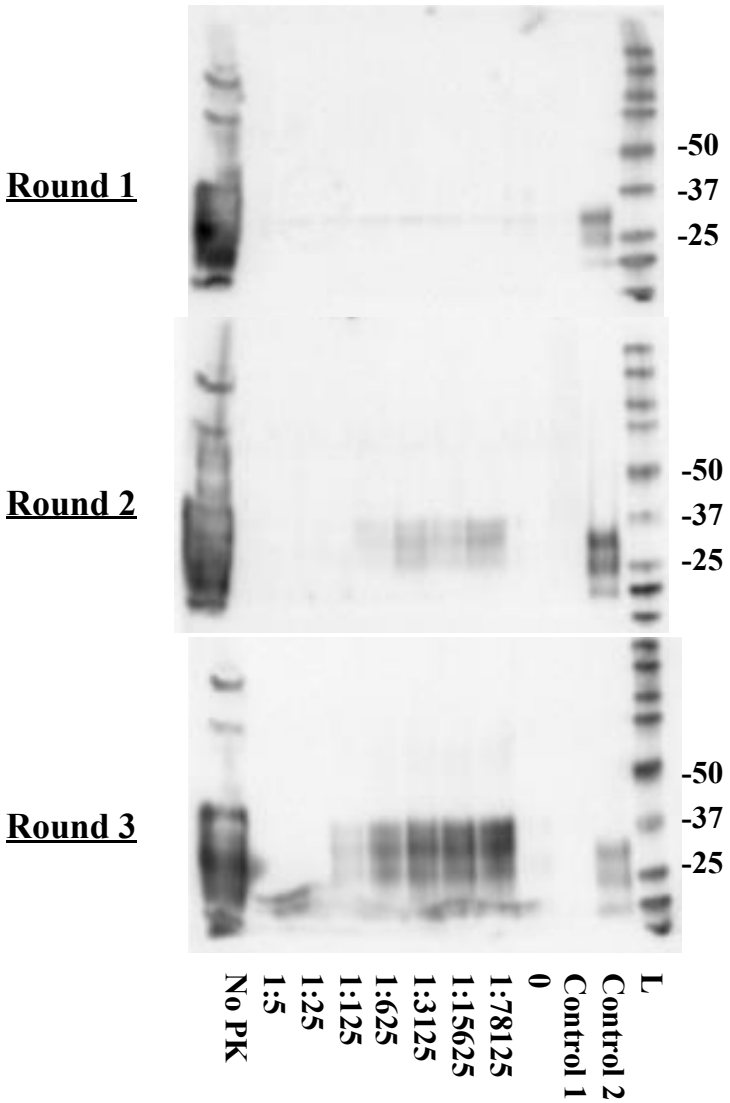

FIGURE 5.3: WESTERN BLOTS SHOWING INHIBITION OF sPMCA AMPLIFICATION OF ELK-CWD AT HIGHER FECAL CONCENTRATIONS (1-10%).

A 10% fecal homogenate sample was spiked with 1% elk infectious brain homogenate and serially diluted into 10% TgElk normal brain homogenate. Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K, "0" – NBH only (no PrP^{CWD} seed), "Control 1" – dilution of a 10% IBH sample stored at -80ºC and not subjected to PMCA, "Control 2" – dilution of a 10% IBH sample in conversion buffer and stored at --80ºC and not subjected to PMCA and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

In two separate fecal homogenate preparations (Fig. 5.4 Panel A and Panel B), by the third round of sPMCA, PrP^{CWD} could be consistently detected in the fecal pellets of experimentally infected WTD, shed at four different post inoculation (PI) time points (Fig. 5.4). For each homogenate preparation, the fecal pellets were obtained from different samples within pens with the same P.I. time point.

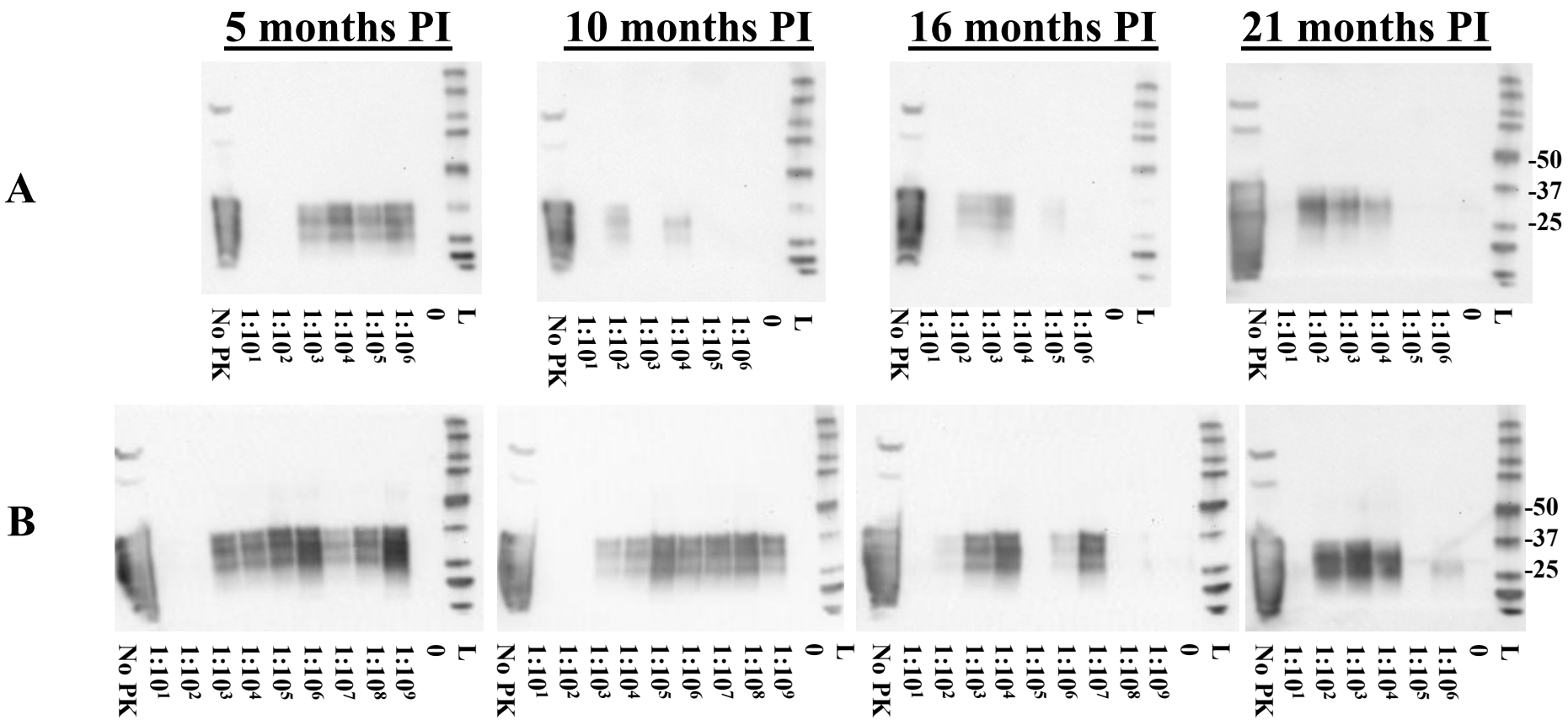

FIGURE 5.4: WESTERN BLOTS FROM THIRD ROUND sPMCA PERFORMED ON 10% FECAL HOMOGENATES PREPARED FROM FECAL PELLETS COLLECTED FROM SEPARATE PENS OF EXPERIMENTALLY INFECTED WTD (5 MONTHS POST INOCULATION [PI], 10 MONTHS PI, 16 MONTHS PI AND 21 MONTHS PI).
Ten percent fecal homogenate preparations were serially diluted (10^1 – 10^9) in conversion into 10% TgElk normal brain homogenate and subjected to three rounds of PMCA. PrP^{CWD} could be detected at all time points (5, 10, 16 and 21 months PI) post inoculation for two separate homogenate preparations (Panel A and Panel B). All zero controls remained negative. Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K, "0" – NBH only (no PrP^{CWD} seed) and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

In the first preparation (Fig. 5.4 Panel A), PrP^{CWD} was found in the highest dilution of $1:10^6$ for the 5-month PI fecal pellets, suggesting relatively high burden of PrP^{CWD} with detection sensitivities extending beyond the maximum 10^6 dilution. The PrP^{CWD} signal was not found in dilutions higher than $1:10^4$ for the 10, 16 or 21-month PI samples for the first set of homogenate samples (Panel A). In the second fecal preparation (Fig. 5.4 Panel B), PrP^{CWD} was found in the greatest dilution of $1:10^9$ for the 5 and 10 month samples once again indicating that further dilutions could have been accommodated compared to the 16 and 21 month PI pellets where the PrP^{CWD} signal could not be found in dilutions greater than $1:10^7$ or $1:10^4$ respectively. For both preparations, all the negative controls remained negative throughout three rounds.

The large differences in signal strength found in the 10 month P.I. fecal homogenate preparations, suggest significant variability in PrP^{CWD} burdens between any two sample preparations, requiring that several repeats of fecal sample homogenates need to be analyzed in order to quantify potential PrP^{CWD} burdens shed within these sample. However, the detection of PrP^{CWD} in fecal samples as early as 5 months PI and as late as 21 months PI suggest that shedding of prions occurs in feces throughout the course of infection and that levels within these excreta can be substantial. For example, the 10 month PI sample represented in Panel B of Figure 5.4 demonstrates that PrP^{CWD} could be measured down to a 10^{-9} dilution after 3 rounds: when considering that a negative fecal sample spiked with a 1% WTD IBH resulted in detecting of PrP^{CWD} at a 10^{-5} dilution in 2 rounds and a 10^{-7} dilution in 3 rounds, this implies that a relatively large concentration of

PrP^{CWD} may exist in feces and consequently shed by infected animals (i.e., equivalent or greater than a 1% IBH spike). This finding is particularly relevant since it is generally believed that under natural exposure conditions relatively low concentrations of PrP^{CWD} coupled with repetitive exposures between animals represents the most likely transmission scenario. However, the data presented in Figure 5.4 suggests that single, high doses exposures may also occur under natural conditions, particularly in the context of fecal samples within the environment. Accidental (i.e., browsing), or intentional (i.e. coprophagy), ingestion of fecal pellets by cervids may result in single high dose exposure scenarios under natural settings, and consequently provides credence to the results and conclusions drawn from exposure studies carried out under laboratory conditions (i.e., high dose). Arguably, patchy distribution of fecal pellets containing high titre PrP^{CWD} in the environment may provide alternate ways for transmission, in addition to those proposed through environmental bioaccumulation (Nichols *et al.*, 2009) or low dose repetitive exposures (Haley *et al.*, 2009a).

5.2.2.2 Urine

Urine samples from CWD infected white-tailed deer collected during post mortems at 2, 4 and 21 months PI, were serially diluted into 10% TgElk NBH for amplification and detection of PrP^{CWD} by PMCA (Fig. 5.5). The PrP^{CWD} signal from the urine collected at 21 months PI was detected in some of the dilutions as early as the second round and was present in all the dilutions up to $1:10^4$ by the third and fourth rounds. Urine samples taken from individual WTD at earlier time

points of two and four months PI also produced detectable signals for some of the dilutions by the second round but unlike the later PI urine samples, not all the dilutions produced a detectable signal by the fourth round suggesting lower starting levels of PrP^{CWD} present in samples from earlier PI time points.

5.2.2.3 Saliva

Samples of saliva collected the day before the post mortem at 21 months PI were serially diluted in TgElk normal brain homogenate (Fig. 5.6). The PrP^{CWD} signal from the saliva of WTD #82 and #83 was detected in some of the dilutions as early as the second round and was present in all the dilutions up to $1:10^4$ by the third and fourth rounds. Negative controls remained negative for both experiments.

The findings in this chapter demonstrate the ability of PMCA to characterize tissue tropism in infected individuals, detect CWD in excreta and secreta from experimentally infected WTD and show that concentrations of PrP^{CWD} shed in the feces of infected animals can be significant, with levels equivalent to that seen in infected brain material. Prion infectivity through secreta/excreta has previously been demonstrated when Safar *et al*, using a transgenic mouse bioassay, with this paper also demonstrating the transmission of scrapie prions using Syrian hamsters that were cohabitated or exposed to the bedding of SHas orally infected with Sc237 prions (Safar *et al*., 2008).

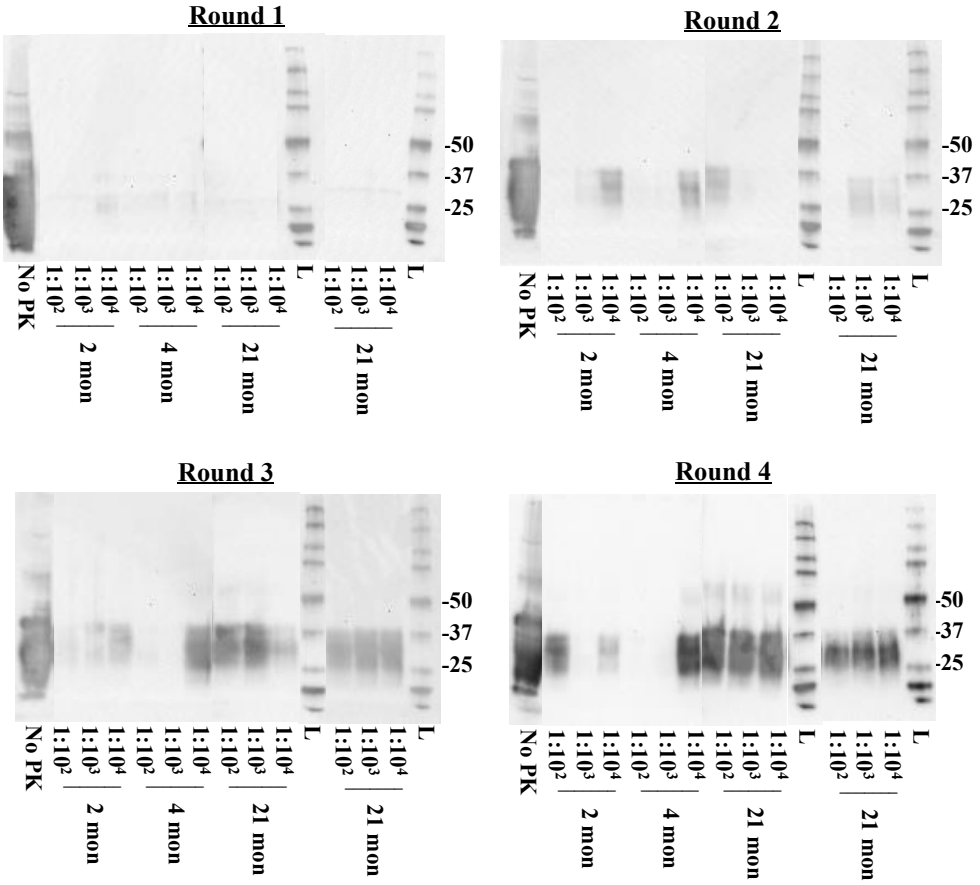

FIGURE 5.5: WESTERN BLOTS SHOWING THE AMPLIFICATION OF PrP^{CWD} FROM URINE SAMPLES COLLECTED FROM EXPERIMENTALLY INFECTED WHITE-TAILED DEER AT DIFFERENT TIME POINTS.

The PrP^{CWD} signal was detectable in some of the dilutions for all three time points as early as the second round. By the fourth round, the 21-month PI urine samples were detectable at all dilutions. Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K and "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown).

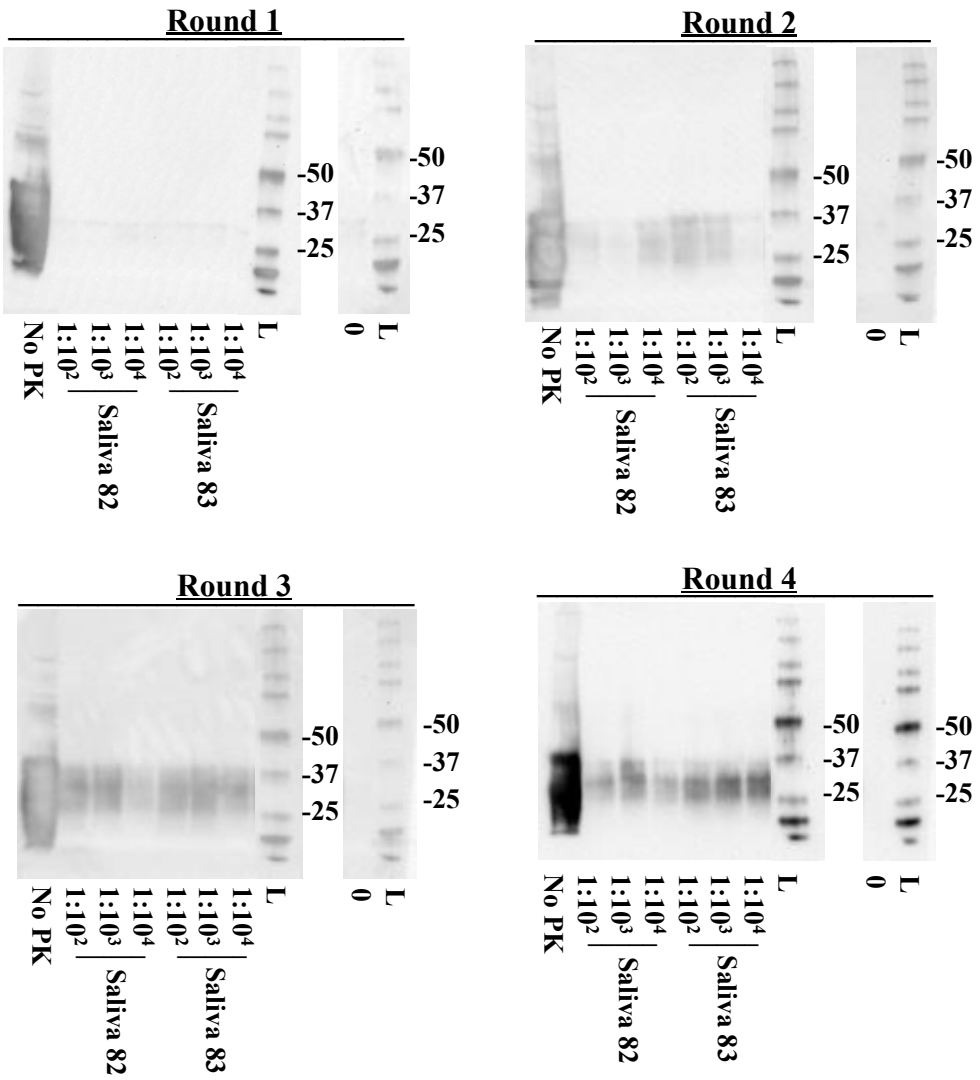

FIGURE 5.6: WESTERN BLOTS SHOWING THE AMPLIFICATION OF PrP^{CWD} FROM SALIVA SAMPLES COLLECTED FROM EXPERIMENTALLY INFECTED WHITE-TAILED DEER.

Saliva samples were collected at 21 months post inoculation (PI). The PrP^{CWD} signal was detectable as early as the second round while the zero control remained negative throughout 4 rounds. Other lanes are designated as follows: "No PK" – NBH not treated with Proteinase K, "L" – molecular weight ladder (50kD, 37kD, and 25kD standards shown) and "0" – NBH only (no PrP^{CWD} seed)

Tamguney *et. al.*, showed that asymptomatic CWD-infected mule deer excrete infectious prions through feces, using intracerebral inoculation of

irradiated deer feces into transgenic mice over-expressing the cervid PrP gene (Tg[CerPrP] mice) (Tamguney *et al.*, 2009). Haley *et al*., (2009a) demonstrated CWD infectivity in concentrated, dialyzed urine and saliva samples from experimentally infected WTD, through i.c. inoculation of transgenic mice expressing the cervid PrP gene (Tg[CerPrP] mice). PMCA was also used to correlate the bioassay data, detecting PrP^{CWD} in urine samples, while traditional assays (Western blot, ELISA) were unable to detect PrP^{CWD} in any of the samples (Haley *et al*., 2009a). Haley *et al*., (2009b) also revealed low levels of CWD prions, again undetectable by traditional tests, in neural and lymphoid tissues of orally exposed deer by both PMCA and bioassay, illustrating the sensitive and specific application of sPMCA in the diagnosis of low-level prion infection (Haley *et al.*, 2009b). Pulford *et. al*. (2012), detected PrP^{CWD} in feces from naturally exposed elk using PMCA, reporting detection of prion loads from 100 to 5000 pg PrP^{CWD}/g of feces and showed for the first time PrP^{CWD} in feces from naturally exposed free-ranging elk demonstrating the potential of PMCA as a new, noninvasive CWD diagnostic tool.

CHAPTER 6 GENERAL DISCUSSION

6.1 Significant findings

The significant findings of this thesis were as follows:

1. *PMCA may be an ultrasensitive laboratory diagnostic tool for detecting CWD in infected ungulates, including white tailed deer, mule deer, elk and red deer.*
2. *PMCA could be used to detect CWD across different PrP genotypes.*
3. *PMCA could be used to characterize tissue tropism of* PrP^{CWD}
4. *PMCA could be used to detect CWD in excreta and secreta from experimentally infected WTD, including feces, urine and saliva.*
5. *Concentrations of* PrP^{CWD} *shed in the feces of infected animals can be significant, with levels equivalent to that seen in infected brain material.*

This chapter discusses the relevance, application and impact of these findings to the field and examines key knowledge gaps that should be considered a focus of future research.

6.2 Overview and Implications of Research Findings

Chronic wasting disease (CWD) is a fatal, highly contagious and environmentally transmitted prion disease. It is the only transmissible spongiform encephalopathy (TSE) known to exist in free ranging and captive animals. Since it

was first identified in 1978 the disease has spread rapidly through cervid populations in Alberta, Saskatchewan and over 16 states in the USA; it is now considered endemic in many of these areas. Apart from the intrinsic value of cervids in our ecosystems and their importance as a subsistence food source in some remote communities, these animals form the basis of a number of economically important industries such as hunting, guiding/ outfitting, wildlife viewing and game farming. Users of deer and elk contribute more than $20 billion annually to agency budgets and local economies (Conner *et al.*, 2008). Their simultaneous value as both a consumptive and non-consumptive resource places additional constraints on the ways cervids can be managed. The contrasting perspectives of managers, hunters, the livestock industry, environmentalists, aboriginals and the general public, along with the difficulty of understanding infectious diseases themselves, can create substantial challenges in the management of chronic wasting disease in North America.

The increased frequency and geographic distribution of CWD in North America, the environmental reservoirs of infectious agent, the resistance of prions to environmental degradation, and the potential for interspecies transmission all raise significant concerns about risks to humans and other animal species. Although no human or natural domestic livestock cases of TSE have been linked to CWD, health risks remain a concern to the general public. Bovine spongiform encephalopathy (BSE or mad cow disease) in cattle and the epidemiological link as a causative agent of variant Creutzfeldt-Jakob disease in humans (Will *et al.*, 1996) raises some concern that a strain of CWD could potentially cross the species barrier into humans. These issues emphasize the need for a better

scientific understanding of environmental contamination and related disease transmission to improve disease management and determine long-term health risks to humans and ecosystems. Currently, management of CWD in free-ranging cervids is focused on eradicating or containing the disease to minimize potential future issues such as spillover to livestock, human health impacts or impacts on free-ranging cervid populations.

CWD prevention measures have been instituted in many jurisdictions, including restrictions on the importation and raising of captive cervids, carcass regulations banning importation of brain or spinal column tissue as well as bans on feeding or baiting stations for cervids (Conner *et al*., 2008). Surveillance programs for the detection of CWD and determining distribution and prevalence vary in different provinces and states but most samples are acquired from hunter-harvests or from outbreak farms. Hunter harvested samples come from regular hunts or special disease management hunts. Other surveillance data comes from cervids culled by professional sharpshooters as part of CWD management programs. Typical management strategies include an increase in harvesting and non-selective culling to reduce animal densities in an attempt to limit the spread of the disease; however, these programs have had minimal impact (Saunders *et al.*, 2012). Testing of captive cervids is routine in most states and provinces, but varies considerably in scope from mandatory testing of all dead animals to voluntary herd certification programs or mandatory testing of only animals suspected of dying from CWD (Saunders *et al*., 2012). Improved methods for detecting low levels of PrP^{CWD} may allow for development of early ante-mortem or more sensitive post-mortem diagnostic tests for CWD, which in turn may

provide better estimates of disease prevalence in both farmed animals and endemic environments (Hill & Collinge, 2003a; Hill & Collinge, 2003b; Spraker *et al.*, 2004; Castilla *et al*., 2004).

6.2.1 PMCA as a potential diagnostic tool for CWD in cervids

The data presented in this thesis highlights the potential utility of PMCA as a rapid diagnostic test for CWD. The PMCA assay was capable of detecting CWD in a diverse range of cervid species (white-tailed deer, mule deer, elk and red deer) and correlated well with traditional diagnostic methods used for CWD detection in WTD. The data demonstrate a high level of sensitivity and specificity for the PMCA assay (see Section 5.2). After one round of amplification, the PrP^{CWD} signal could be consistently detected across four different species of cervids (white-tailed deer, elk, mule deer and red deer) showing a PrP^{CWD} signal increase of at least four to six $\log_{10}$ over the original infected brain homogenate. When comparing traditional diagnostic test methods (ELISA, immunohistochemistry and Western Blot) to PMCA, all animals deemed CWD positive by traditional methods were shown to be PMCA positive. For traditional diagnostic methods, three tests (ELISA, IHC, and WB) across two different tissues (obex and RLN) were required for confirmation of CWD infection (i.e., in some cases the obex was negative whereas RLN was positive), whereas the PMCA assay confirmed the presence of PrP^{CWD} in either the obex or RLN in these CWD infected animals. The ability of PMCA to detect CWD in infected animals of different *Prnp* genotypes was also shown, supporting the theory that a

glycine/serine polymorphism at codon 96 of the *Prnp* gene in WTD appears to confirm some level of resistance to CWD in this species (O'Rourke *et al*., 1999). Experimentally-infected white-tailed deer that were *S/S* homozygous were found to have lower levels of PrP^{CWD} suggesting they were more resistant to CWD infection compared to *G/S* heterozygotes and *G/G* homozygotes. In all cases, two rounds of PMCA were needed to amplify the lower levels of PrP^{CWD} from *S/S* genotypes whereas only one round was required to amplify PrP^{CWD} from *G/G* genotypes indicating a higher initial burden. For *G/S* heterozygotes, ELISA optical density values were intermediate, and either one or two rounds of PMCA were required to amplify PrP^{CWD} in these animals.

Furthermore, based on the data presented in this thesis, conventional diagnostic tests (ELISA, IHC and Western blot) may potentially underestimate disease prevalence in a CWD outbreak when compared to PMCA. This conclusion is based on the observation that a proportion of animals that were negative for CWD by traditional diagnostic methods were shown to be positive by PMCA in the obex and/or retropharyngeal lymph nodes, but all animals originated from outbreak farms known to house CWD infected animals. We currently do not know whether animals that are positive by PMCA but negative by a conventional test panel (ELISA, IHC and Western blot on RLN tissues) will eventually die of CWD disease if removed from an infected environment. Based on experimental infection studies, animals that become positive for PrP^{CWD} by conventional testing of RLN tissues will eventually succumb to the disease (Sigurdson *et al*., 1999). However, we do not know whether animals exposed under *natural* conditions (e.g., animals residing on a CWD-infected farm) that test negative for CWD by

conventional tests, will eventually succumb to the disease (i.e., CWD incubating at levels below the current sensitivity levels of conventional tests). Understanding the diagnostic sensitivity of assays and their correlation to disease outcomes is critical for managing CWD in both farmed and wild cervid populations. There are few published studies evaluating the effect of management intervention on CWD. This is a result of the disease being relatively recently described, monitored and managed, combined with CWD's long disease course and relatively slow spatial spread. Currently, there is contention in the control strategies used for farms implicated in a CWD outbreak: do all animals have to be destroyed or only the infected population? The basis for decision-making rests in designating an animal as being 'infected' or 'non-infected' and consequently the sensitivity of the diagnostic assay and its correlation with long-term health outcomes for an animal. At present, the data presented in this thesis supports the requirement to cull all animals within an outbreak farm, based on the observation that some animals appear to be harboring CWD below the detection limits for traditional methods but detectable by PMCA. It is possible that implementation of highly sensitive tests could be used to detect PrP^{CWD} in infected animals before they become highly contagious to other animals. These infected animals, once identified, could be quarantined and monitored away from the other healthy cervids before they infect them as opposed to eradicating the entire herd.

Extrapolating this to native cervid populations, CWD surveillance programs that rely on conventional tests may considerably under-estimate infectivity levels in native cervid populations. In the limited dataset presented in this thesis, upwards of 25% (i.e., 4/12 animals) appeared to be harboring CWD based on

sPMCA and below the detection limits for conventional tests. This has extremely important implications in the management of CWD in the wild, and for ecological models that predict current rates and spread of disease outside endemic areas and that are based on sensitivity levels of traditional diagnostic test results. We are currently applying the PMCA assay to detection of CWD in hunter-kill harvests in Alberta, Canada, as a comparative assessment of conventional tests against PMCA for determining the true prevalence of CWD in an endemic area (L.M.Price unpublished data).

6.2.2 Tissue tropism of CWD in WTD based on PMCA

The semi-quantitative nature of the PMCA assay was useful at estimating burdens of PrP^{CWD} in different tissues of infected white-tailed deer (see Section 5.2.1). The data suggested that while some infectivity may be present in muscle tissue at later stages of the disease, the burden of infectivity was significantly lower than the burden found in the internal organs. It appeared that PrP^{CWD} was present early after infection and remained at high levels throughout the course of the disease for organs such as the spleen, kidney, liver, adrenal gland and colon. Like striated muscle, deposition of PrP^{CWD} in cardiac muscle did not occur until later stages of the disease (16 months post inoculation), but unlike striated muscle the presence of PrP^{CWD} in cardiac muscle remained at high levels throughout the remaining course of the disease (see Section 5.2.2). The only striated muscle samples to test positive for PrP^{CWD} came at 16 months post inoculation whereas all samples before and after this time point tested negative suggesting, a) either a

spike in PrP^{CWD} deposition at this particular time point, b) variability in PrP^{CWD} content within the sample may be due to quantity of nervous tissue within each biopsy sample, and/or c) that the burden of PrP^{CWD} deposited in striated muscle is far less than other tissues.. PrP^{CWD} has been associated with the sciatic nerve (Sigurdson *et al.*, 2001), which is the largest peripheral nerve found in mammals, running down the hind legs from the lower back. If any part of this nerve were accidently collected with the muscle sample and homogenized together, there is the potential for a positive PMCA result to occur within a striated muscle biopsy. Nevertheless, there is still significant nervous innervation within individual muscle fibers comprising striated muscle, suggesting that in general, PrP^{CWD} content in striated muscle is low. The data suggest that the dose of exposure may increase considerably when certain tissues such as the spleen, heart, liver and kidney are consumed and when compared to the muscle. Others have reported evidence of PrP^{CWD} in the nervous system, the lymphoreticular system, the hematopoietic system, skeletal and cardiac muscles, pancreas, fat, retina, and the adrenal and salivary glands of naturally and/or experimentally infected animals through bioassay (Sigurdson & Miller, 2003; Sigurdson, 2008; Seelig *et al.*, 2010; B. Race *et al.*, 2009; Spraker *et al*., 2010; Imran & Mahmood, 2011) raising concerns regarding the consumption of these internal organs by hunters or others who consider them to be delicacies.

PrP^{CWD} has been found in skeletal muscle of mule deer through i.c. inoculation of Tg(CerPrP) mice (Angers *et al*., 2006) and in WTD by WB that indicated the concentration of PrP^{CWD} in skeletal muscles of CWD-infected WTD was approximately 2000-10,000-fold lower than in brain tissue, while revealing

its seeding ability through PMCA (Daus *et al*., 2011). No other study has been able to report the presence of PrP^{CWD} in skeletal muscle of any other cervid species (elk, moose or caribou), which may suggest the levels of PrP^{CWD} in deer species are higher in skeletal muscle than the PrP^{CWD} levels found in elk, moose and caribou, but in general appear to be comparatively low in all cervid species compared to other tissues and organs. There have been reports of differing tropism between elk and deer where PrP^{CWD} levels were found to be lower in lymphoid tissues of elk compared to WTD and MD (B. L. Race *et al*., 2007) whereas in cardiac muscle, PrP^{CWD} was detected in elk and WTD through ELISA, WB and IHC, but not found in MD or moose (Jewell *et al.*, 2006)

If certain organs are harboring significantly high doses of PrP^{CWD}, consumers should be made aware of this. While consumption of skeletal muscle has had no evidence contributing CWD to humans, other organs may provide much higher exposures, providing more opportunity for the combination of events to occur that allows the species barrier to be crossed. Dose levels of this magnitude may not be accounted for in the current models predicting the spread of CWD, its management and its effect on human populations. Human surveillance/epidemiological programs need to account for this potential behavioral risk factor since hunters who consume only skeletal muscle may have less exposure risk to CWD compared to their counterparts who also consume internal organs as delicacies. The implementation of a policy to dissuade the public from consumption of the delicacies that pose a higher risk may be required.

6.2.3 Shedding of PrP^{CWD} in secreta and excreta of infected animals

The development and application of PMCA to excretory/secretory samples (i.e., feces, urine and saliva) from cervids may also help address other important questions related to the epidemiology of CWD: when do animals become contagious during the course of infection, what are the PrP^{CWD} burdens associated with shedding and if they are pre-symptomatic, how long has an animal been infected? The work presented in this thesis demonstrate the utility of using PMCA to detect prions in feces, urine and saliva from WTD with promising results (see Section 5.2.2), and in using these methods in comparative assessment of the diagnostic potential in experimentally-infected, farmed and free-ranging WTD populations. Infectious CWD prions have recently been identified in feces, saliva, blood and urine of infected cervids (Pulford *et al*., 2012; Mathiason *et al*., 2006; Mathiason *et al*., 2009; Tamguney *et al*., 2009; Haley *et al.*, 2009) using animal bioassay, and PMCA has been successfully used to identify PrP^{CWD} in many of these samples as well (Pulford *et al*., 2012; Haley *et al*., 2009a).

Detection of prions in feces from experimentally infected cervids collectively suggests that PrP^{CWD} may be shed in excreta early after infection and continue throughout the course of the disease (Chapter 5). Variation in fecal consistency, found even throughout the day, may account for the wide discrepancies of PrP^{CWD} found among fecal samples. Shedding and turnover of epithelial cells in the gut is high, and consequently the shedding of cellular constituents of gut associated lymphoid tissue (GALT) may also be very high

(i.e., due to high fiber content, hair, and soil as part of the diet). PrP^{CWD} is known to invade the GALT shortly after infection (Fox *et al.*, 2006) and so a single fecal pellet composed of large or small numbers of epithelial cells from an infected animal may lead to considerable variation in PrP^{CWD} concentrations in individual fecal pellets. It was found that in one fecal sample, the PrP^{CWD} concentration was equivalent to a 1% concentration of WTD IBH, representing a substantial burden of shedding in this tissue not only within a single fecal sample, but potentially throughout the course of an infected animals lifetime. Based on the following assumptions: 1) that an infected cervid will defecate several times in a day (i.e., 3 times/day with each defecation episode accounting for 250 g (Arthur & Alldredge, 1980), 2) that based on extremely high turnover of epithelial cells in the gut (Creamer *et al.,* 1961), at least one of these episodes results in deposition of high titre PrP^{CWD} equivalent to 1% IBH, 3) that an animal sheds prions throughout its infected lifetime (Tamguney *et al.,* 2009) 4) that an infected animal may live for 1.5 years before dying of disease (i.e., 450 days) (Williams, 2005), and 5) that the average brain size of a WTD is 500g (Logan & Clutton-Brock, 2013) , then the burden of shedding is estimated to be equivalent > 2 infectious brains being spread throughout each infected cervid's environment (i.e., [250g/day x 450 days]/ 500g/brain x 1% = 2.25 brains). This is important, since it is generally believed that exposures under natural conditions are most likely a result of low dose, repetitive exposures of naïve animals to PrP^{CWD} (Nichols *et al*., 2009; Haley *et al*., 2009a). Although Haley *et al.* and Tamguney *et al.* demonstrated infectivity in saliva, urine and fecal samples (Haley *et al*., 2009a; Tamguney *et al*., 2009), the samples required concentration of PrP^{CWD} and i.c.

inoculation to cause infection , suggesting that although infectious prions are shed, the concentrations required to establish infection are low and, a) may accumulate in the environment to levels needed to establish infections in naïve animals, or b) that repetitive low dose exposures results in eventual doses sufficient for disease causation. The data presented in this thesis suggests that infected animals may occasionally shed high concentrations of PrP^{CWD} and consequently naïve animals may consume single large doses of PrP^{CWD} under natural conditions (i.e., accidental ingestion of fecal pellets).

In addition to the shedding of PrP^{CWD} in feces, PrP^{CWD} appeared in urine samples collected at 2, 4 and 21 months post inoculation (Chapter 5). Due to the low sample size and variability associated with urine concentration found throughout the day no inferences of quantitation could be made other than their presence throughout the course of infection. It is known however that as terminal stages approach, clinical symptoms show a large increase in water intake resulting in more frequent and larger volumes of urination (Williams, 2005). This could imply that the spread of PrP^{CWD} into the environment becomes more intense the longer the disease has to progress giving another reason for the importance of identifying infected individuals as soon as possible.

PrP^{CWD} was found in saliva samples collected at 21 months post inoculation, which may coincide with hypersalivation; commonly seen near the terminal stages of CWD (Williams, 2005). Factors that concentrate cervids in localized areas such as salt licks or artificial feeding points in residential/rural areas may facilitate transmission if saliva, containing PrP^{CWD}, is frequently deposited for oral uptake by subsequent visiting naïve cervids.

It is unknown whether the presence of PrP^{CWD} in various excretory/secretory discharges is a biological response in a futile attempt to remove the fatal agent from the body, but their presence and potentially differing burdens at various time points throughout the course of the infection, may be insightful into predicting how long a cervid has been infected. While this thesis, given its limited number of samples, cannot conclude with any statistical significance whether the burden of shedding was higher or lower at certain PI time points as compared to others, an interesting study would be to determine whether any time trends involving shedding take place. If it could be determined, for instance, that shedding in feces is consistently greater soon after infection rather than later, as the disease disseminates from areas such as the gut associated lymphoid tissues where shedding could occur, towards areas deeper inside the host like the central nervous system where shedding would not be accessible, the information could be used to predict how long an asymptomatic individual has been infected. This would be useful in not only identifying infected individuals but also, based on how long an individual has been infected, useful in mapping out the true scope and spread of the disease and predicting which directions the epidemic will head to next.

The findings presented in this thesis also have important implications for understanding the management of CWD. Arguably, environments may be contaminated with high concentrations of PrP^{CWD} (feces [this thesis], decomposing carcasses [Miller et al]) etc., and that movement of infected animals with their home (or migratory) ranges can result in the 'patchy' dissemination of high concentrations of prions throughout the environment. Congregation areas

(i.e., over wintering zones) may represent foci of high concentrations of prions through both high dose fecal deposits and subsequent bioaccumulation over time. Minimizing congregation in endemic areas may help reduce transmission burdens and slow the spread, but ultimately the spread of the disease may depend on infected animals moving throughout their host range and depositing sporadic high levels of PrP^{CWD} in feces. Arguably, the spread of CWD may depend less on bioaccumulation of low doses of PrP^{CWD} in the environment (i.e., saliva and urine) but rather on the patchy distribution of high concentrations of PrP^{CWD} throughout an environment as a result of fecal deposition.

6.3 Future Research Considerations

Our data (Sections 3.2.2.5 and 5.2) demonstrate a high level of sensitivity and specificity of the PMCA assay for detection of prions in multiple species, tissues and excreta/secreta of cervids. Collectively, it suggests that PrP^{CWD} may be shed early after infection and throughout the course of infection through different routes. These different routes may be useful in identifying infected individuals and estimating a time frame for when the animal became infected. This information would prove useful in multiple ways: mapping out the true extent of the current epidemic, determining how well captive cervids serve as models for their free-ranging counterparts, assist in addressing issues of contagion and help to define what constitutes an infectious dose.

6.3.1 Application of PMCA as a potential diagnostic tool for CWD in cervids

Most of the information regarding CWD biology and disease ecology comes mainly from research on captive animals, therefore, much remains unknown about the disease course in free-ranging animals. It is unknown how well captive cervids serve as models for their free-ranging counterparts in this regard. To what degree CWD would reduce population growth in nature or change the demographics in a biologically significant way, still need to be addressed. Transmission parameters such as the length of the infectious period, route and rate of transmission and the contribution and intensity of the environmental effect still need to be determined in free-ranging cervid populations. The presence of prions in secreta and excreta has been shown to be infectious but to what degree are these samples infectious in a natural setting? Are the doses found in nature truly infectious or, like in many experiments, are they required to be concentrated? As previously discussed, conventional thinking currently suggests that sample doses found in nature are most likely much lower but delivered repetitively as compared to the large doses found in experimentally inoculated subjects delivered over a short period of time. The experimental routes of inoculation also differ vastly with oral inoculation, the most likely path for naturally infected individuals, at one end of the spectrum and direct intracranial injection at the other end producing different infection success rates as well as pre-patent periods. High dose experimentally-infected animal models cannot answer whether repetitive low doses can cause an animal to die from CWD before the end of its natural life.

Both multiple repetitive low dose experiments and single high dose experiments from relevant excreta samples (i.e., feces) that reflect the natural exposure conditions are required to answer these knowledge gaps. Contaminated bedding as an environmental substitute could be dosed out through a titrated infectious homogenate delivered to an absorbent matrix allowing observation and measurement of the route and rate of infection. Information relating to the accuracy of computer models that incorporate only repetitive low dose exposure estimates could be obtained. Our data suggest the possibility of repetitive high dose exposures in nature, equal to what is found in the brain, based on levels of PrP^{CWD} found in experimentally infected WTD fecal pellets (Section 5.2.2.1). In this context future research should examine the infectivity of fecal pellets with high levels of PrP^{CWD} comparable to experimental infectious brain homogenates, through oral inoculation studies. These experiments could be used to demonstrate that single or repetitive high dose exposure from the environment is possible especially considering that cervids consume significant levels of soil or through accidental ingestion of fecal material.

Unfortunately, the low levels of PrP^{CWD} infectivity associated with the early presymtomatic animals would be undetectable by traditional detection methods. The specificity and ultrasensitivity of the PMCA method developed in this thesis could potentially find these answers; however, infectivity trials are still required to validate these findings. It would be interesting to determine whether the amplified product from excreta/secreta is infectious to cervids through bioassay and whether the product has the same biophysical characteristics as the original inoculums (PK sensitivity, vaculation patterns in infected animals, etc) to show

faithful replication or show evidence of strain differentiation. Samples 121-14, 12-49, 12-60 and 12-80 from our farmed WTD study (Section 4.4) were positive by PMCA, but suspect or negative by traditional diagnostic methods. These samples, in addition to the amplified products from fecal, urine and saliva studies need to be inoculated into cervidized transgenic mice or corresponding cervid species in order to prove authenticity of the infectious nature of prions shed in these tissues.

Currently it is not known whether the same infectious agent is responsible for all of the CWD outbreaks found in the wild and on farms across Canada and the USA in various cervid species exhibiting different genotypes or if multiple strains of PrP^{CWD} involved. There is strong evidence that distinct CWD strains exist (Angers *et al*., 2010), distinguished by varied incubation periods, clinical symptoms, PrP^{CWD} conformations and CNS PrP^{CWD} depositions (B. Race *et al*., 2009). Intraspecies and interspecies transmission of prions from CWD-positive deer and elk isolates resulted in identification of at least two strains of CWD in rodent models (Angers *et al*., 2010), indicating that CWD strains likely exist in cervids (Saunders *et al*., 2012).

Nothing is currently known about the natural distribution and prevalence of CWD strains; however, the PMCA protocols developed from this thesis were able to detect PrP^{CWD} in farmed and naturally occurring outbreaks across Alberta and Saskatchewan, in multiple cervid species and even within a single species exhibiting genotypic variation (i.e., *Prnp* genotype). A careful analysis of the glycosolation patterns in conjunction with IHC to determine PrP^{CWD} deposition and a comparison of incubation periods and clinical symptoms may help

determine whether the PMCA PrP^{CWD} assay is able to detect PrP^{CWD} in multiple strains and help identify and define these strains in the future.

6.3.2 Instrumentation recommendations

The PMCA method developed in this thesis was capable of detecting a $1:10^5$ dilution of 10% CWD infectious brain homogenate from different species in one 18 hour round, but improvements to the sonicator set up can still be made to accommodate a larger number of samples sites, with improved reliability for each sample. As it stands, only about 25 samples can be processed at a time and when rounds take 18 hours serially diluted 3 to 4 times, high through-put is compromised. The sonicator used to deliver the pulse that breaks apart the forming PrP aggregate into smaller seeding units is not ideal. The surface of the sonicator horn does not provide a uniform distribution of power. There appears to be a power gradient from the center outwards resulting in more power being delivered to the middle of the horn as compared to the outer edges. The only way to keep samples relatively uniform is to form a ring around the center so each sample is the same distance from the middle of the horn in order to receive the same delivered power. This significantly reduces the number of samples that can be effectively processed in each round. Calibration of the horn using a determined dilution of infectious brain homogenate into normal brain homogenate that is known to amplify to the level where a consistent signal can be found should be applied to all sample sites to determine a zone of effectiveness where conditions are such that amplification may occur. The use of sand or salt to visualize the

wave patterns on the horn surface during sonication would also be useful in determining the best and most consistent sample placements and power settings for optimal amplification. A tube holder designed with these specifications in mind would be far more useful than the tube holder currently supplied in Misonix's PMCA package. The current tube holder implies that 177 samples may be processed at a time where in fact it is more like 20.

Improvements to the overall design would be conceptually analogous to the thermal cycler used in the polymerase chain reaction (PCR) in order to create uniform conditions for all samples sites. Each sample's position would have its own sonicating tip similar to the way a thermal cycler has its own heated position for each sample. The way the sonicator is designed now would be similar to trying to perform PCR using a candle as the heat source and placing samples all around it in multiple rows and expecting all the samples to receive the same amount of heat. The samples closer to the center would receive far more heat than the ones furthest out and with a technique as temperature dependant as PCR this would not work. An upside down 96-tip horn replacing the current microplate horn may provide a more uniform distribution of delivered power. Placing the sample tubes in a more suitably designed tube rack suspended in a water bath so that each tube is slightly above each individual sonicator tip would prevent any breach of containment. Sample tubes would remain closed and sonication would occur through the tube as before. A lid on top of the tubes and a sample rack with slightly larger holes so that the sample tubes hang equally by their lids would also ensure a uniform distance between the sonicating tips and the tubes similar to what is found in almost all PCR thermal cyclers.

6.4 Conclusion

Given that the routes of PrP^{CWD} transmission are still uncertain, managerial decisions for the disease's eradication based on the supervision of animal trade and quarantine or mandated destruction of affected herds may appear less promising. A contaminated environment providing repetitive low to high doses may make this goal impossible. Therapeutic intervention targeting molecular mechanisms involved in the pathogenesis of the disease, possibly in the form of wildlife vaccines, may be a better substitute for the control of CWD. In addition, *Prnp* polymorphisms S96G, M132L and S255F in cervids have been associated with resistance to CWD. Selective breeding, at least in the case of captive animals, may be helpful in disease management, though the issue of multiple strains has prevented this from solving the scrapie problem (Dawson *et al.*, 2008).

Finally, an important and unique issue for Canada is the spread of CWD into caribou populations in northern Canada. Recently it was reported that CWD had been experimentally transmitted from white-tailed deer to reindeer demonstrating for the first time that a sub-population of reindeer are susceptible to CWD by oral inoculation implicating the potential for transmission to other *Rangifer* species (Mitchell *et al*., 2012). The long migration routes and large densely packed herds of caribou would allow rapid and extensive dissemination of the disease. Indigenous human populations within these regions rely heavily on wildlife as a food resource. Infection of caribou with CWD would also increase exposure of indigenous populations to CWD through consumption of wild meat

allowing the potential for interspecies transmission as was seen during the “mad-cow” outbreak less than 10 years ago.

Appropriate development of the methods described in this thesis (see Sections 3, 4 and 5) could lead to the reliable identification of infected individuals earlier in the development of the disease reducing the impact, providing more accurate data for epidemiological models and allowing better management of the disease to be achieved.

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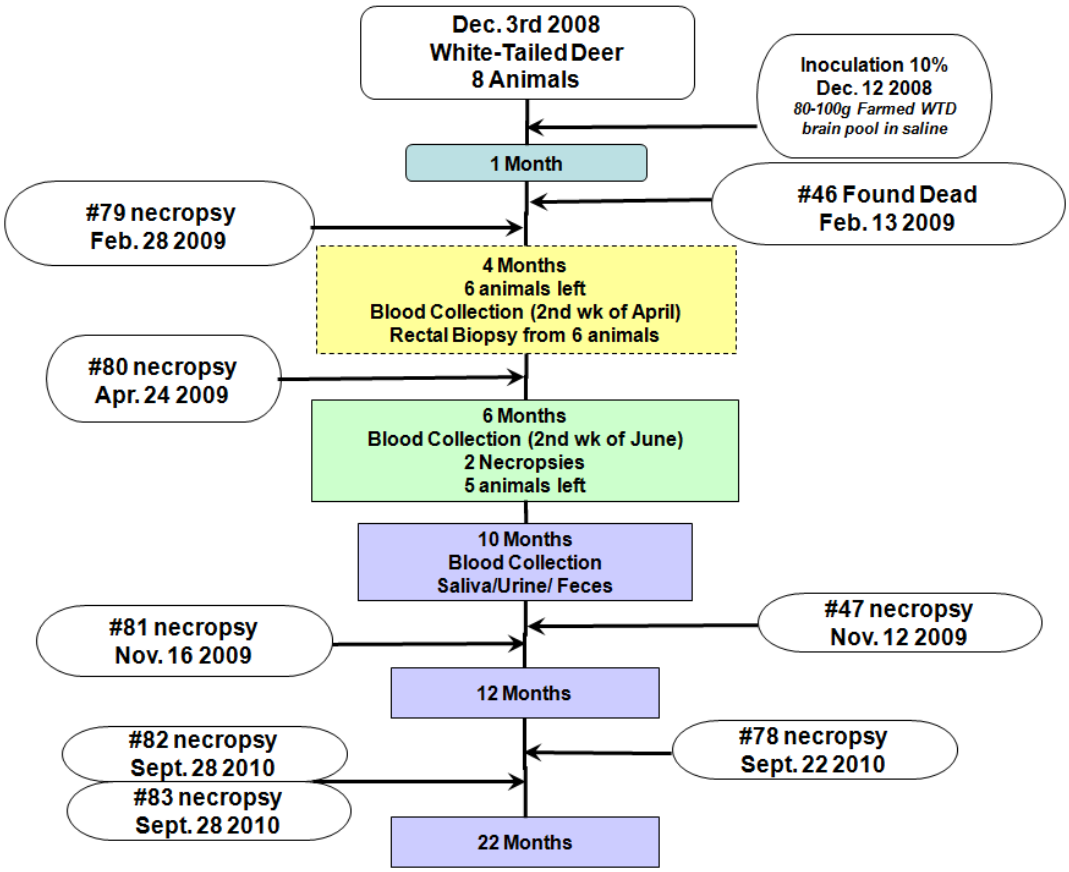
CHAPTER 8 APPENDIX

A: Overview of the CFIA experimental infection study of CWD in WTD.



B: Summary of diagnostic outcomes of WTD experimental infections with CWD.

Summary of WTD-CWD Project 28

10 Animals Acquired (2 died en route)

Animal #	Inoculation Date	Genotype	Post-mortem	Clincal	ELISA RESULTS		IHC	
46	2008-12-12	GG	Found Dead 2009-02-13	NO	Br Ln Tons	- - -	Br Ln Tons	- - -
79	2008-12-12	GG	2009-03-03	NO	Br Ln Tons	- + -	Br Ln Tons	 + -
80	2008-12-12	GG	2009-04-22	NO	Br Ln Tons	- + +	Br Ln Tons	- + +
47	2008-12-12	GS	Euthanized 2009-11-12	NO	Br Ln Tons	+ + +	Br Ln Tons	+ + +
81	2008-12-12	GG	2009-11-16	NO	Br Ln Tons	+ + +	Br Ln Tons	+ + +
82	2008-12-12	GG	2010-09-28	YES	Br Ln Tons	+ + +	Br Ln Tons	+ + +
83	2008-12-12	GG	2010-09-28	YES	Br Ln Tons	+ + +	Br Ln Tons	+ + +
78	2008-12-12	GG	2010-09-22	YES	Br Ln Tons	+ + +	Br Ln Tons	+ + +