DISSERTATION

CHRONIC WASTING DISEASE: A MODEL FOR PRION TRANSMISSION VIA SALIVA AND URINE

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY NICHOLAS JAMES HALEY ENTITLED CHRONIC WASTING DISEASE: A MODEL FOR PRION TRANSMISSION VIA SALIVA AND URINE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHRONIC WASTING DISEASE: A MODEL FOR PRION TRANSMISSION VIA SALIVA AND URINE

Chronic wasting disease (CWD) of cervids is a prion disease distinguished by its high level of transmissibility, wherein bodily fluids and excretions are thought to play an important role. Typical of all prion diseases, CWD is characterized by the forced conversion of the normal prion protein (PrP^{C}) into a misfolded isoform (PrP^{CWD}), which has been shown to accumulate primarily in tissues of the lymphoid and nervous systems, though has also been found in other peripheral tissues including elements of the cardiovascular, musculoskeletal, and urogenital systems. Despite strong evidence that natural infection is acquired from the environment, as well as saliva and blood, a more thorough evaluation of excreta, including saliva, urine, and feces, is essential for a comprehensive foundation for (1) understanding how environmental CWD-contamination occurs, (2) developing in vitro assays for the antemortem identification of CWD-infected cervids, and (3) demonstrating the pathogenesis of the disease in the natural host.

In this dissertation, two approaches are used to identify infectious CWD prions and PrP^{CWD} in the bodily fluids and tissues of CWD-exposed white-tailed deer: a novel bioassay system using a transgenic mouse line expressing the cervid PrP protein (Tg[CerPrP] mice), and a recently developed prion amplification assay known as serial

protein misfolding cyclic amplification (sPMCA). In conjunction with immunohistochemistry and western blotting, these two assays were used to definitively identify CWD prions in saliva and urine, in addition to elements of the lymphoreticular system, central and peripheral nervous systems, and urogenital and oropharyngeal tissues.

In initial experiments, concentrated urine and saliva samples from terminal CWD+ white-tailed deer, suspected of harboring infectious CWD prions, was assessed by Tg[CerPrP] bioassay and sPMCA. Authentic prion infectivity was detected in urine and saliva using both detection systems in the case of urine, though only mouse bioassay successfully demonstrated CWD prions in saliva. The concentration of abnormal prion protein in bodily fluids was very low, as indicated by: undetectable PrP^{CWD} levels by traditional assays (western blot, ELISA) and prolonged incubation periods and incomplete TSE attack rates in inoculated Tg[CerPrP] mice. These findings helped to extend the understanding of CWD prion shedding and transmission and portend the detection of infectious prions in body fluids in other prion infections.

Based on the identification of CWD prions in saliva ("prionsialia") and urine ("prionuria"), I next sought to determine whether deer previously exposed orally to urine and feces from CWD+ sources, while conventional test-negative, may actually be harboring very low level CWD infection not evident in the 19 month observation period in initial cervid bioassay studies. A selection of tissues, including those of the lymphoreticular and both central and peripheral nervous systems were fully examined, initially using Tg[CerPrP] bioassay to demonstrate true infectivity, and secondarily with sPMCA. Positive controls consisted of issues from CWD+ deer exposed orally to saliva; negative control tissue sets were collected from deer exposed orally and intracranially to

CWD-negative brain. PrP^{CWD} was detected in the tissues of orally exposed deer by both sPMCA and Tg[CerPrP] mouse bioassay; each assay revealed very low levels of CWD prions previously undetectable by western blot, ELISA, or IHC. Serial PMCA analysis of individual tissues identified that obex alone was positive in urine/feces exposed deer. PrP^{CWD} was amplified from both LRS and neural tissues of positive control deer but not from the same tissues of negative control deer. Detection of subclinical infection in deer orally exposed to urine and feces (1) suggests that a prolonged subclinical state can exist such that observation periods in excess of two years may be needed to detect CWD infection, and (2) illustrates the sensitive and specific application of sPMCA in the diagnosis of low-level prion infection.

Despite the confirmation of infectious prions in urine and saliva, along with conventional test-negative deer exposed to urine and feces, the manner in which infectivity is transferred to these excreta is unknown. To address this, I went on to apply sPMCA to tissues associated with production and excretion of urine and saliva in an effort to seek proximal sources of prion shedding. I blindly analyzed oropharyngeal and urogenital tissues, reproducibly demonstrating PrP^{CWD} in each tissue examined in 3 rounds of sPMCA; whereas blood samples from the same animals and concurrent negative controls remained negative. Tissue distribution was affected by route of inoculation and CNS burden. The identification of PrP^{CWD} in bodily fluids and conventional-test negative tissues – in the absence of detection by conventional methods – may indicate the presence of protease-sensitive infectious prions in excretory tissues not revealed by assays employing PK digestion or other means to remove PrP^{C} reactivity.

The continued evaluation of bodily fluids and peripheral tissues via sPMCA may therefore allow additional insights into prion transmission, trafficking, and pathogenesis.

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I am grateful to everyone in the CSU Retrovirus and Prion Research Lab, but would especially thank Davis Seelig for being a true friend and constant source of ideas – good and bad – over the past 6 years. Candace Mathiason provided crucial assistance with many aspects of the studies described from provision of tissues to being the impetus behind refining my assay technique and efficiency, and Nate Denkers offered endless distractions from the day-to-day experiences of graduate work. Jeannette Hayes-Klug, Kelly Anderson, and Sheila Hayes deserve immense credit for their expertise in laboratory animal-care and their ability to provide a continuous supply of transgenic mice for my work. Without David Osborn and Sallie Dahmes, the deer studies upon which most of my theses are based would not have been possible. I would like to extend thanks to Glenn Telling and the members of his laboratory, notably Tanya Seward and Shawn Browning, for their invaluable roles in the creation of the transgenic mice used in these studies.

I would next like to acknowledge the members of my graduate committee, including Gary Mason, Mark Zabel, and Eric Ross. All provided great insight and intellect into the design and interpretation of these studies. Without Gary, in particular, I believe the mundanity of academia would have been overwhelming. Mark and members of the Zabel lab were integral in assisting with the development and refinement of the sPMCA assay, while Eric was always a committee member I could depend on for his promptness and excellent insight into the alternate yeast prion universe.

I would also like to thank a number of faculty members at CSU, including former microbiology residents Kristy Pabilonia and Andy Torres. Both were incredibly supportive through the course of my residency and embarkation into research. Although our experience together was brief, my residency under the guidance of Hana Van Campen was invaluable for my understanding of real-world microbiological applications and the necessity of persistence and hard work.

The National Park Service, specifically Jenny Powers and Margaret Wild, have been tremendously helpful in their collaboration, providing invaluable tissue and bodily fluid samples from “real world” CWD cases in Rocky Mountain National Park. It is my hope that this collaborative effort continues, as their resources are second to none.

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DEDICATION

This work is humbly dedicated to my mentor, Edward Hoover. His guidance, support, intellect, and patience over these long 5 years have helped to transform me from an unfocused veterinarian with a genuine interest in research into something resembling a bona fide scientist.

I would also like to dedicate this work to my parents Robert and Chip Haley, who instilled in me the ideals of hard work and dedication, and I am forever indebted to them for pushing me out of the nest as quickly as they could.

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INTRODUCTION

The prion diseases:

Prion diseases – transmissible spongiform encephalopathies or TSEs - are infectious, progressive and uniformly fatal neurodegenerative diseases of humans and animals. The designation "prion disease" is based on the association of these diseases with aggregates of a conformationally altered and post-translationally modified isoform of the normal cellular prion protein (PrP^{C}) (Williams 2005). In the course of disease, the normally α-helical rich prion protein, which is a detergent-soluble, protease-sensitive, glycophosphatidylinositol (GPI) anchored membrane-bound sialoglycoprotein, is converted into a detergent-insoluble, protease-resistant, β-sheet rich isoform (Weissmann and Flechsig 2003). Low molecular weight aggregates of this isoform, typically defined by a 27-30kDa protease-resistant "core" protein demonstrable by western blotting, have been shown to correlate strongly with both infection and disease (Bolton, Rudelli et al. 1991; Race, Jenny et al. 1998; Tzaban 2002; Silveira, Raymond et al. 2005). The studies described in this dissertation are framed in the context of the most widely accepted paradigm for the etiology of TSE's: the protein-only hypothesis, in which it is postulated that TSE's occur as a result of infection with the relatively protease-resistant isoform, PrP^{res}, that propagates by translating its altered biophysical characteristics to native PrP^{C} (Griffith 1967; Soto and Castilla 2004). The misfolded isoform has been designated variously, including PrP^{Sc} (scrapie-associated abnormal prion protein), PrP^{res} (protease-resistant abnormal prion protein), and PrP^{d} (disease-associated prion protein). In this dissertation, the term "PrP^{res}" will be used to describe term infectious prion proteins in a general sense, while the expression conventionally applied to CWD prions, "PrP^{CWD},"

will be used to denote the infectious prion protein associated with chronic wasting disease (CWD). Apart from iatrogenic transmission of human prions via tissue transplantation, dentistry, and blood transfusion, surprisingly little is known about the natural routes of prion transmission, though accumulating research points to a strong role for excreta, including saliva, urine, and feces (Hadlow, Eklund et al. 1974; Williams and Young 1992; Miller and Williams 2004; Seeger, Heikenwalder et al. 2005; Kariv-Inbal, Ben-Hur et al. 2006; Mathiason, Powers et al. 2006; Murayama, Yoshioka et al. 2007; Safar, Lessard et al. 2008). This latter aspect of prion pathogenesis, transmission as modeled by the naturally occurring prion disease of cervids, CWD, is the subject of studies described in this dissertation.

Molecular biology of the prion protein:

The cellular prion protein, PrP^{C} (Figure I-1), is an approximately 209 amino acid protein serendipitously discovered by Prusiner and colleagues in 1982 while searching for molecular markers associated with TSE infection in hamsters (Bolton, McKinley et al. 1982; Collins, Lawson et al. 2004). It was later identified in a wide range of species, from fish (Favre-Krey, Theodoridou et al. 2007) to birds (Gabriel, Oesch et al. 1992), to turtles (Simonic, Duga et al. 2000). The protein is expressed in a number of organ systems, though is greatest in neuronal tissues (Collins, Lawson et al. 2004). The physiologic function of the prion protein is unknown, though has been proposed to be involved in heavy metal binding, specifically copper transport (Viles, Cohen et al. 1999). The protein is a product of the PRNP gene, which, in humans, is found on chromosome

20 and is approximately 15.4kb in length (Prusiner 1998). After translation, the protein may be modified through the addition of various carbohydrate groups at one of two potential C-terminal locations (human PrP residues 181 and 197), a disulfide bond (linking human PrP cysteine residues 179 and 214) along with a C-terminal GPI anchor as mentioned above (Rudd, Merry et al. 2002). Addition of carbohydrate groups results in three predominant forms of the prion protein identifiable on western blotting: an unglycosylated isoform of approximately 27kD, a 29kD monoglycosylated isoform, and a 36kD diglycosylated isoform (Segarra, Lehmann et al. 2009). The N-terminus of the protein contains an octapeptide repeat region and is relatively unstructured, while the C-terminus has a more defined, α-helix-rich structure (Prusiner 1998). Several polymorphisms have been identified in the human prion protein, which may confer either resistance or susceptibility to prion infection. Of these, a methionine – valine polymorphism at human residue 129 has been shown to be important in sporadic, iatrogenic, and BSE-linked CJD (Goldfarb, Brown et al. 1994; Windl, Dempster et al. 1996; Zeidler and Ironside 2000).

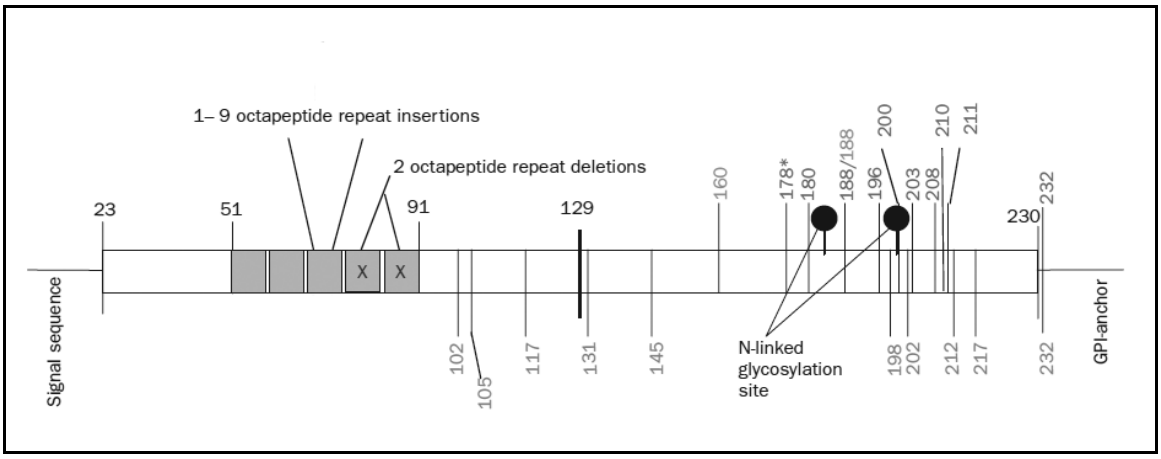

Figure I-1. Primary structure of the human prion protein. Locations of various regions contributing to disease susceptibility or resistance, including octapeptide repeats and various amino acid polymorphisms, are indicated. Adapted from Collins, Lawson, and Masters. (Collins, Lawson et al. 2004)

In cervids, the expressed prion protein is composed of approximately 232 amino acids, and, like other species, has three distinctly glycosylated isoforms (Jewell, Conner et al. 2005; Kelly, Mateus-Pinilla et al. 2008). The predominant isoform in cervids is diglycosylated, with the mono- and unglycosylated isoforms less abundant to varying degrees. As with humans and other prion-susceptible species, the prion protein of mule and white-tailed deer has a number of polymorphisms. Emerging as the most important of these, relative to susceptibility, is residue 96, which may be either glycine or serine (Johnson, Johnson et al. 2003; Jewell, Conner et al. 2005; Meeker, Ye et al. 2005; Haley, Seelig et al. 2009). A number of other residues have also been shown to be variable in cervids, including residue 132 in elk (Green, Browning et al. 2008), 209 in moose (Baeten, Powers et al. 2007), 138 in caribou (Happ, Huson et al. 2007), and 226 in Sika deer (Meng, Zhao et al. 2005), though the significance of these polymorphisms and their relationship to susceptibility is unknown. The cervid prion protein sequence shares an

approximately 89% amino acid identity with the human prion protein; this difference is thought to be significant enough to confer human resistance to infection with cervid-origin infectious prion proteins (Kong, Huang et al. 2005).

Origins of, and cell types involved in, prion shedding and horizontal transmission:

Saliva and urine have been shown to play major roles in the transmission and diagnosis of a number of emergent infectious diseases, from HIV to henipaviruses (Ewing, Scorpio et al. 1994; Ruhl-Fehlert, Brem et al. 2000; Ersin, Kocabas et al. 2004; Aguilar-Setien, Loza-Rubio et al. 2005; Amado, Villar et al. 2006; Saito, Alfieri et al. 2006; Slots, Saygun et al. 2006; Pant Pai 2007; Wacharapluesadee and Hemachudha 2007). Alarmingly, though, the role of these bodily fluids in prion diseases has not been thoroughly evaluated. Prior to the research described in this study, the true role of urine and saliva in CWD transmission, as well as its origins in these fluids, was unknown. It still remains to be shown if infectious PrP^{res} is associated with cells in these bodily fluids, as prion proteins – either proteolytically cleaved from the cell membrane or present in exosomal fractions (Parkin, Watt et al. 2004; Robertson, Booth et al. 2006; Zhao, Klingeborn et al. 2006) – may be found in cell-free preparations of excreta (Gatti, Metayer et al. 2002; Yakovleva, Janiak et al. 2004; Maddison, Whitelam et al. 2007). *In situ* and bioassay studies have identified PrP^{res} in lingual nerve fibers, epithelial cells, taste buds, and salivary glands, any or all of which may be involved in prionsialia (Hadlow, Eklund et al. 1974; Mulcahy, Bartz et al. 2004; DeJoia, Moreaux et al. 2006; Vascellari, Nonno et al. 2007). Immune effector cells that have been implicated in prion

replication, e.g. lymphocytes, have been demonstrated in salivary preparations (Shugars, Slade et al. 2000; Shugars, Patton et al. 2001), and could be involved as carriers in the natural transmission of prion diseases. Epithelial cells, the predominant cell type present in saliva (Xie, Onsongo et al. 2007), have also been hypothesized to be involved in prion disease transmission(Pammer, Suchy et al. 1999; Pammer and Tschachler 2002), though there is no evidence for its role in prionsialia. In renal tissues, protease-resistant prion protein has been localized to the renal papillae, collecting ducts, and ectopic lymphoid tissue in scrapie and CWD models (Hamir, Kunkle et al. 2006; Siso, Gonzalez et al. 2006; Ligios, Cancedda et al. 2007), while we have found PrP^C heavily expressed in cervid transitional epithelial cells of the urinary bladder and urethra. Any of these tissues and cell types may be involved in prionuria to some extent, though it has also been postulated that leukocytes – specifically dendritic cells – excreted during the course of nephritis, may be the source of infectivity (Seeger, Heikenwalder et al. 2005; Murayama, Yoshioka et al. 2007). Definitive identification of the fluids, tissues, and associated cell type(s) involved in the natural routes of prion transmission will provide valuable information applicable to the pathogenesis, transmission, and diagnosis of prion diseases.

Natural history of Chronic Wasting Disease:

CWD was first recognized as a distinct disease of captive mule deer in Colorado in 1967 (Williams and Young 1992). Identification of CWD as a TSE was made on the basis of the characteristic microscopic brain lesions 11 years later, in 1978 (Williams and Young 1980). In the time since its initial discovery and description, CWD has been identified in

captive and free-ranging cervid populations in Wyoming, Nebraska, Montana, Oklahoma, South Dakota, Wisconsin, Minnesota, Kansas, New Mexico, Utah, Illinois, West Virginia, New York, Michigan, Virginia, Missouri, Alberta, Saskatchewan, and Korea (Sohn, Kim et al. 2002). CWD is unique among prion diseases in that it is the only TSE known to infect free-ranging populations and is aggressively transmitted among and between cervid species (Williams and Young 1992; Miller, Williams et al. 2000). The natural routes of CWD transmission have yet to be fully elucidated, though infection has been demonstrated following exposure to contaminated environments (Miller, Williams et al. 2004), and more importantly to both blood and saliva from CWD-infected deer (Mathiason, Powers et al. 2006). Though urine and feces were not initially shown to transmit infection, the sensitivity of transgenic mouse bioassay and protein misfolding cyclic amplification have retrospectively allowed for the identification of CWD prions in both inoculated animals and in urine itself (Haley, Mathiason et al. 2009; Haley, Seelig et al. 2009). On this basis, and with an incomplete scientific underpinning, every state, Canadian province, and member of the European Union have implemented strict importation restrictions on live cervids, their byproducts and biological fluids (European Parliament; House; Resources; www.promedmail.org; 2007). The identification of prions in bodily fluids, once thought not to occur, has profound impact on our understanding of prion transmission and biocontainment.

Human exposure to CWD:

It is evident that CWD has been present in wild cervid populations of northern Colorado and southern Wyoming for at least 40 years, though potentially there has been a low level of endemnicity for much longer (Miller, Williams et al. 2000). The prevalence of CWD varies depending on species and locale, but the most recent data indicates that it can be as high as 30% in some areas of Colorado, and much higher in captive populations (Williams 2005; Keane, Barr et al. 2008; Patton, Swanson et al. 2008). As surveillance efforts intensify, CWD continues to be identified in areas previously thought to be free of infection, including recent discoveries in a number of states east of the Mississippi River. Thus it is probable that substantial human exposure has occurred in the growing endemic area since its discovery. Human exposure to CWD may occur directly, as occurs when hunters, game processors, and wildlife agents field dress, butcher, collect samples from, preserve, dispose of, or consume cervids and their products. Recent cases have shown that the number of individuals that come in contact with an infected deer may range from a single person and their family, as was described in a study evaluating the likelihood of CWD-associated CJD infection in individuals who consumed venison in Wisconsin (2003), to as many as 350 individuals, as occurred during a sportsmen's feast in New York (www.promedmail.org; Garruto, Reiber et al. 2008). In Colorado, over 320 hunter-killed deer, elk, and moose were identified as CWD positive by the Colorado Department of Wildlife between 2005 and 2007 (CDOW 2007), while in Wisconsin, over 1030 CWD positive hunter-killed animals have been identified since the disease's initial discovery (WDNR 2007). At minimum, these figures translate to thousands of individuals directly exposed to CWD over the past half-decade, with potentially tens of thousands more

exposed following consumption or handling. Because of the likelihood of environmental transmission and persistence of CWD, human exposure to CWD in the environment may also occur; however, there are no data available by which to estimate this level of exposure.

Potential association of human neurologic disease with CWD:

With the apparent transmission of BSE to humans after consumption of infected material, and the expanding distribution of and human exposure to CWD, concerns over the possible zoonotic transmission of CWD to humans are growing (Bosque 2002). At present, there is very little information to support or refute a link between CWD and human neurologic diseases, while its clinical manifestations in humans are difficult to predict. Three investigations into eight cases of atypical CJD in the United States could not rule out the potential for a role of CWD in human illness, as experimental studies, (e.g. infectivity assays or glycoform analysis) were not performed (Belay, Gambetti et al. 2001; 2003; Belay, Maddox et al. 2004; Xie, O'Rourke et al. 2006; Anderson, Bosque et al. 2007). A study in transgenic mice expressing the human prion protein failed to demonstrate susceptibility of these animals to CWD infection, although a very limited number of animals, genotypes, and CWD isolates were examined without the benefit of secondary passage experiments (Kong, Huang et al. 2005). To more completely evaluate a link between CWD and human neurologic disease, continued retrospective analysis of atypical CJD cases, as well as prospective studies following individuals known to have consumed or handled CWD infected materials, need to be performed (Garruto, Reiber et

al. 2008). Other emerging diagnostics, including protein-misfolding cyclic amplification, may prove a useful surrogate whilst these studies are undertaken (Soto, Saborio et al. 2002; Kurt, Perrott et al. 2007; Haley, Mathiason et al. 2009).

Economic impact of CWD:

Estimating the present or potential economic impact of CWD across the nation is difficult given the absence of research specifically evaluating the impact of CWD on local, state, or national economies. Several factors must be considered when attempting to estimate CWD impact, including 1) economic losses from unsold hunting licenses, equipment, and related expenditures, 2) the estimated value of natural resources embodied by cervid populations and the actual value of captive animals, and 3) cost of resources diverted to containment, management, and eradication of CWD (Seidl 2003; 2007). As an example, the Wisconsin Department of Natural Resources estimates it has spent over $20 million since 2002 on surveillance, management and eradication efforts, and plans to spend an additional $5 million yearly toward management (Rolley 2005). Others have speculated that Wisconsin's potential economic loss, as a result of lost hunting-related expenditures alone, approaches $100 million *annually* (Bishop 2002). These costs, however, are trivial when compared to the estimated $1 billion dollar impact deer hunting has on Wisconsin's annual economy, or the estimated $22.6 billion spent yearly across the country on hunting-related activities (Rolley 2005; 2007). These estimates of course do not take into account the national and international economic impact should a single instance of trans-species CWD transmission to cattle or humans occur.

Development of in vitro prion detection assays:

The level of infectious prions in early infection and various non-neural tissues and bodily fluids has been demonstrated to be beyond the limits of detection in traditional assays, e.g. western blotting, immunohistochemistry, and ELISA (Head, Kouverianou et al. 2005; Mathiason, Powers et al. 2006). For this reason, a diagnostic aide with sensitivity approaching that of polymerase chain reaction (PCR) has long been sought. In 2001, a technique was identified which seemed to approach the power of PCR in amplifying extremely low levels of PrP^{res} seed in a sample (Saborio, Permanne et al. 2001). This assay, known as protein-misfolding cyclic amplification, or PMCA (Saborio, Permanne et al. 2001; Soto, Saborio et al. 2002; Soto, Anderes et al. 2005), has been used in the detection of prions in a number of TSE's, including hamster models of scrapie, bovine spongiform encephalopathy in cattle, vCJD in humans, and CWD (Castilla, Saa et al. 2005; Soto, Anderes et al. 2005; Pastrana, Sajnani et al. 2006; Saa, Castilla et al. 2006; Jones, Peden et al. 2007; Kurt, Perrott et al. 2007; Murayama, Yoshioka et al. 2007; Murayama, Yoshioka et al. 2007; Weber, Giese et al. 2007; Haley, Mathiason et al. 2009; Haley, Seelig et al. 2009). PMCA takes advantage of the conversion of the normal cellular prion protein, PrP^{C}, by a seed of infectious prion material, *in vitro*. (Figure I-2) Suspect samples are incubated with aliquots of normal brain homogenate (NBH), interspersed with short bursts of sonication. In the most commonly employed method, thirty minutes of incubation is followed by a 40-second period of sonication; this constitutes one cycle of amplification (Saborio, Permanne et al. 2001; Castilla, Saa et al. 2005; Castilla, Saa et al. 2006; Haley, Mathiason et al. 2009; Haley, Seelig et al. 2009). Typically, samples undergo roughly 96 cycles of amplification over 48 hours, which

comprises a single round of PMCA. The homogenates may then be either analyzed by western blot, or serially transferred to a new preparation of NBH for further rounds of amplification (sPMCA). Recently, a limitation of sPMCA in a scrapie model has been reported – i.e. the apparent stochastic "spontaneous generation" of protease-resistant prion material following many rounds of amplification. The distinction between contamination and spontaneous generation has been argued and can of course be problematic (Agrimi, Cosseddu et al. 2008; Soto, Barria et al. 2008), which serves to emphasize the paramount importance of appropriate controls in all PMCA experiments, especially where unknown samples are being assayed (Deleault, Harris et al. 2007).

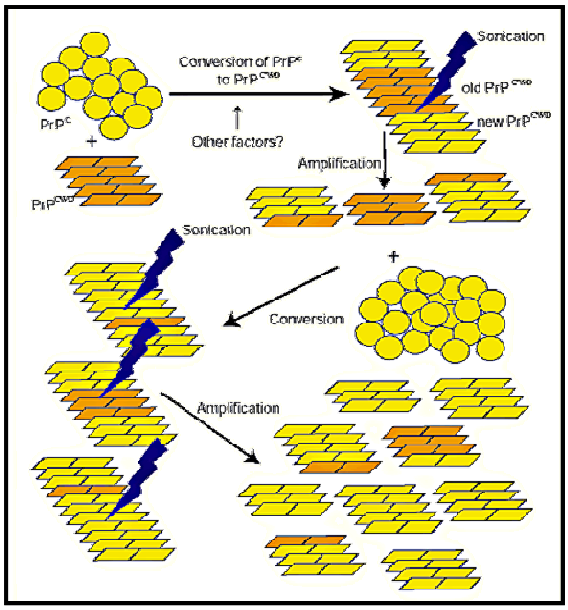

Figure I-2: Schematic overview of PMCA (adapted from Saborio et al. and Telling). (Saborio, Permanne et al. 2001; Telling 2001)

A number of other promising assays are currently under development in various research institutions around the world. While it has not received the widespread attention that PMCA has, a method known as quaking-induced conversion or "QUIC" also has

strong potential for *in vitro* diagnostic capabilities (Atarashi, Moore et al. 2007). To date, not much is known about the specificity of the assay, though the sensitivity is comparable to that of sPMCA (Ryou and Mays 2008). The conformation-dependent immunoassay (CDI) is another assay showing promise in the sensitive and specific differentiation of infectious prion proteins (Safar, Scott et al. 2002; Safar, Geschwind et al. 2005); while a number of other diagnostics relying on differential conformation and substrate binding are under continual development. (Reviewed by Aguzzi et al. (Aguzzi, Heikenwalder et al. 2004))

Questions surrounding CWD upon which this dissertation is focused:

This thesis seeks to address several unanswered questions uncovered during the course of research into CWD transmission, including: (1) what is the role of urine and saliva in the facile transmission of CWD in nature, (2) could a developing prion amplification assay, sPMCA, be used to identify CWD prions in tissues and bodily fluids of CWD-exposed cervids, and (3) what are the proximal sources of CWD prions in bodily fluids? To address these questions, I first evaluated the infectious potential of cervid urine and saliva in a transgenic [CerPrP] mouse bioassay. I next developed and optimized an *in vitro* prion amplification assay (sPMCA) for the detection of conventionally imperceptible levels of PrP^{CWD} in clinical samples and used this assay to probe bodily fluids and tissues for amplifiable CWD prions. It is my belief that the studies described herein enhance our current knowledge of CWD transmission and

pathogenesis, and provide an alternative to lengthy mouse bioassay experiments for the detection of low-level prion concentrations.

Dissertation Research:

The above background of TSE and CWD research formed the basis upon which the specific aims of this dissertation are based. My first objective was to evaluate urine and saliva samples from terminal, CWD+ deer in both a sensitive Tg[CerPrP] bioassay as well as the newly developed sPMCA assay. Though cervid-origin saliva was previously shown to harbor infectious CWD prions in cervid bioassay, the susceptibility of Tg[CerPrP] mice to saliva was unknown. Conversely, cervid bioassay failed to demonstrate infectious prions in urine from infected deer; the potentially enhanced sensitivity of Tg[CerPrP] bioassay may therefore prove more useful for identifying CWD prions in urine. An optimized serial PMCA assay, on the other hand, could preclude the need for lengthy and expensive mouse bioassay of these fluids altogether. To address this first objective, I inoculated groups of mice with pooled saliva or urine from terminally-infected deer and allowed them to progress to terminal disease. At that time, all mice were evaluated for definitive TSE infection using specific IHC, western blotting, and sPMCA. Concurrently, the pooled saliva and urine samples were assessed for amplifiable PrP^{CWD} by sPMCA. I hypothesized that both transgenic [CerPrP] bioassay and sPMCA would be able to demonstrate PrP^{CWD} in saliva as well as urine.

My findings in the first objective – that both urine and saliva conferred infection in mouse bioassay experiments and that sPMCA could amplify PrP^{CWD} in urine – led me to reconsider the negative results originally obtained in cervid urine and feces bioassay

experiments. Perhaps the dose of CWD prions in urine or feces was too low to result in infection following oral inoculation; alternatively the initial study deer were subclinically infected and were sacrificed prior to fully developing clinical and pathological signs of infection. Therefore, my second objective was to thoroughly evaluate these deer for evidence of CWD infection using the enhanced sensitivity of Tg[CerPrP] bioassay and sPMCA. I hypothesized that the enhanced sensitivity of mouse bioassay and sPMCA would reveal that these deer were truly infected with levels of PrP^{CWD}, below the level of conventional detection assays, and that the centripetal dissemination of CWD prions to the central nervous system would parallel that of conventionally CWD+ animals exposed orally to saliva.

Finally, while I was able to conclusively demonstrate transmissible CWD prions in urine and saliva, as well as in deer orally exposed to urine and feces, the manner in which infectivity arises in these excreta remains unknown. For this reason, the third objective of this work was to investigate tissues involved in urine and saliva production and excretion by using serial PMCA to evaluate salivary gland, tongue, kidney, ureter, and urinary bladder for amplifiable PrP^{CWD}. I hypothesized that PrP^{CWD} could be amplified in these tissues, and that tissue distribution patterns would correlate with both route and source of inoculation as well as apparent central nervous system burden.

The results of this work demonstrate (1) the key role of both urine and saliva in the facile transmission of CWD in nature, (2) the responsibility of various organs and tissues in the centripetal spread of CWD to the central nervous system and the shedding of infectious prions into these fluids, and (3) the utility of both a transgenic [CerPrP] mouse bioassay system and a novel amplification assay – sPMCA – in the sensitive

detection of low levels of PrP^{CWD}. For these reasons, it is my belief that this work provides important advances in the contemporary knowledge of both CWD and prion disease research.

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Chapter 1:

Detection of CWD Prions in Urine and Saliva of Deer by Transgenic Mouse Bioassay

ABSTRACT

Chronic wasting disease (CWD) is a prion disease affecting captive and free-ranging cervids (e.g. deer, elk, and moose). The mechanisms of CWD transmission are poorly understood, though bodily fluids are thought to play an important role. Here we report the presence of infectious prions in the urine and saliva of deer with chronic wasting disease (CWD). Prion infectivity was detected by bioassay of concentrated, dialyzed urine and saliva in transgenic mice expressing the cervid PrP gene (Tg[CerPrP] mice). In addition, PrP^{CWD} was detected in pooled and concentrated urine by protein misfolding cyclic amplification (PMCA). The concentration of abnormal prion protein in bodily fluids was very low, as indicated by: undetectable PrP^{CWD} levels by traditional assays (western blot, ELISA) and prolonged incubation periods and incomplete TSE attack rates in inoculated Tg(CerPrP) mice ($373^{\pm}$3days in 2 of 9 urine-inoculated mice and $342^{\pm}$109 days in 8 of 9 saliva-inoculated mice). These findings help extend the understanding of CWD prion shedding and transmission and portend the detection of infectious prions in body fluids in other prion infections.

BACKGROUND

Chronic wasting disease (CWD) is an efficiently transmitted prion disease of cervids (e.g. deer, elk, and moose) and is the only prion disease affecting free-ranging, non-domestic animals. The origins of CWD are uncertain, but the disease has been present in wild cervid populations of northern Colorado and southern Wyoming for at least 40 years (Williams and Young 1980; Williams and Young 1982). Since its discovery, CWD has been identified in captive and free-ranging cervids in 15 states, 2 Canadian provinces, and Korea (Sigurdson 2008). As surveillance efforts have intensified, CWD has been detected in areas previously thought to be free of infection, including recent discoveries in West Virginia, New York, and Michigan. The prevalence of CWD varies across North America, but can be as high as 30% in some areas of Colorado (Williams 2005).

The mechanisms of CWD transmission are not well understood, although there is evidence that infection is transmitted horizontally and can be acquired from environmental sources (Miller, Williams et al. 2004; Mathiason, Powers et al. 2006), which underlies the assumption that shedding of infectious prions must be significant. With the expanded recognition of the disease across the continental United States, it is also likely that substantial human exposure has occurred. Nevertheless, because of an apparently strong species barrier (Kong, Huang et al. 2005) and the as yet incompletely understood natural routes and kinetics of CWD transmission, the magnitude and consequence of this exposure remain speculative.

Infectious CWD prions have been detected in saliva and blood, e.g. “prionsialia” and “prionemia,” suggesting a role for specific body fluids in transmission and dissemination (Mathiason, Powers et al. 2006). In these bioassay studies in deer, infectivity in urine and feces could not be demonstrated, seemingly at odds with indirect evidence for environmental persistence of CWD prions (Williams and Young 1992; Miller, Williams et al. 2004). In that the presence of prions in body fluids, once thought not to occur, now has impact in understanding of prion transmission and biocontainment, we have continued and extended our investigation of this subject with the present studies.

MATERIALS and METHODS

Cervid sources:

Samples of urine and saliva were collected at terminal disease from five experimentally infected white-tailed deer. Deer in this cohort had been inoculated intracranially with brain, intravenously with blood, or orally with saliva from CWD-infected deer. All source deer were in the terminal stages of CWD infection, and demonstrated moderate to severe neurologic signs, paradoxical polyphagia with declining body condition, polydypsia and polyuria, and were confirmed CWD+ by western blot and immunohistochemistry, as previously described (Mathiason, Powers et al. 2006). Histopathologic examination revealed mild nephritis in all the source deer. In some cases, these changes were age related, while in a single case there was appreciable evidence of pyelonephritis. Four of five the deer were homozygous for glycine at amino acid 96 of the cervid prion gene, while one deer was heterozygous at that location, with alleles encoding for both glycine and serine (Mathiason, Powers et al. 2006). All animals were maintained in accord with Colorado State University IACUC guidelines.

Cervid PrP transgenic mice:

Tg[CerPrP] line 1536 (*tg1536*) mice were generated in the Telling laboratory at the University of Kentucky (Browning, Mason et al. 2004). All mice were screened at weaning for the presence of the [CerPrP] construct by conventional and real-time PCR. All mice testing negative for PrP^{CWD} at the completion of bioassay studies were

rescreened to reconfirm the presence of cervid PrP gene. Animals were treated according to Colorado State University guidelines.

Study samples and preparation:

A 1% w/v homogenate of brain from a single CWD-positive mule deer (provided by Dr. Michael Miller, Colorado Division of Wildlife) was used as the positive control material. A 1% w/v homogenate of brain from a single CWD-negative white-tail deer from outside the CWD-endemic zone was used as a negative control and was provided by David Osborne, University of Georgia. Urine and saliva samples were collected from five symptomatic whitetail deer as described above.

Spiked positive and negative control samples consisted of 1% homogenates of positive or negative deer brain prepared using 10ml of negative control saliva or urine as diluent. For the study groups, 10mls of either urine or saliva were collected from each of five symptomatic deer, 50ml total volume, and homogenized. All urine and saliva homogenates were then prepared for bioassay by lyophilization followed by resuspension in 0.1 volumes of phosphate-buffered saline (PBS) (e.g. a 10-fold concentration) and dialyzed against 2000 volumes of PBS to return the sample to isotonicity.

Mouse bioassays:

Mice were anesthetized with ketamine and xylazine and inoculated intracerebrally into the left parietal lobe with 30µl of inoculum. Incubation time was defined as the number of days from inoculation to the onset of clinical neurological signs consistent

with a TSE (Carlson, Kingsbury et al. 1986). Animals were euthanized when either a symptomatic TSE or signs of distress were evident. Brains were harvested at necropsy and divided longitudinally, with one half prepared for evaluation by western blotting and PMCA, while the remaining half was fixed in 10% neutral-buffered formalin for histopathology and immunohistochemistry.

Western blotting (WB):

Brain tissue was initially prepared as a 10% (w/v) suspension in homogenization buffer (150mM NaCl, 5mM EDTA, and 1%[v/v] triton-X 100 in PBS). Eleven µl of sample homogenate were mixed with 7µl of sample buffer (0.1% [v/v] triton-X 100 and 4%(w/v) SDS in PBS) and digested with 2µl proteinase-K at 500µg/ml (final concentration: 50µg/ml) for 20' at 37ºC followed by 10' at 45ºC. Seven µl of 4X running buffer were then added to the sample, followed by denaturation for 5' at 95ºC. Twenty µl of this preparation were run on a pre-cast 12% SDS-PAGE gel (Invitrogen) in a Bio-Rad electrophoresis apparatus for 2 hours at 110mV. Samples were then transferred to a PVDF membrane for 1 hour at 110mV in a Bio-Rad transfer apparatus. PVDF membranes were subsequently blocked for 1 hour in 5%(w/v) powdered milk in TBST, followed by application of the primary antibody, BAR224-HRP, diluted 1:20,000 in 5% powdered milk in TBST, for 1 hour. Following washing, immunoreactivity was detected using an enhanced chemiluminescent detection system (ECL-plus, Amersham Biosciences) in an LAS 3000™ imaging system. (Fuji Photo Film, Fuji Inc, Valhalla, NY)

Histopathology and Immunohistochemistry (IHC):

Cervid renal tissues and *tg1536* neural tissues were fixed in formalin overnight, treated with 88% formic acid for one hour, washed in tap water for two hours, and then stored in 60% ethanol prior to paraffinization. Paraffin-embedded tissue sections (6µm) were mounted onto positively charged glass slides, deparaffinized, and rehydrated through graded ethanol. Tissues were subjected to Heat Induced Epitope Retrieval (HIER) using an automated antigen-retrieval system (Retriever™) and a proprietary buffer solution (DakoCytomation Target Retrieval Solution, DAKO, Hamburg, Germany). Tissues were then stained with an automated immunostainer, using PrP monoclonal antibody BAR-224 conjugated to HRP as the primary antibody (1:250 final dilution). Detection was completed using HRP-mediated hydrogen peroxide immunostaining (AEC+, DAKO), with haematoxylin as a counterstain.

Serial Protein Misfolding Cyclic Amplification (sPMCA):

Source inocula as well as mice negative for PrP^{CWD} by both WB and IHC were further analyzed by PMCA. The amplification protocol, described below, was similar to those described by Soto and colleagues (Saborio, Permanne et al. 2001; Soto, Saborio et al. 2002). Normal brain homogenate (NBH), the substrate for prion conversion *in vitro*, was prepared in a room that had not previously been used for prion research as follows: naïve *tg1536* mice were euthanized intraperitoneally with 15mg of sodium pentobarbital and perfused with 25ml of 5mM EDTA in PBS via intracardiac catheterization. The calvarium was removed and the entire brain excised and placed on ice. Brain homogenate was then prepared at a 10% (w/v) solution in PMCA buffer (1% triton-X 100

[v/v], 5mM EDTA, 150mM NaCl, and 0.5% saponin [w/v] in PBS adjusted to a pH of 7.2) with the addition of Complete Protease Inhibitors (Roche Pharmaceuticals, Indianapolis, IN) in a dounce homogenizer. Homogenates were then centrifuged for 1 minute at 2000rpm to remove bulk brain material, and the supernatant frozen in single-experiment aliquots at –70ºC in a "prion-free" room until use in PMCA. Experimental handling protocols were identical to protocols commonly used for PCR: NBH was added to a plate in a room not previously used for prion research and then transferred to a room used exclusively for prion research, where samples were added in a biosafety hood prior to sonication. Twenty-five µl of either source inocula or WB and IHC negative brain homogenate was added to 25µl of NBH in individual wells of a 96 well PCR plate (USA Scientific, Ocala, FL), placed in an ultrasonic processor (Misonix, Farmingdale, NY) and incubated at 37ºC. Samples were sonicated for 40s at power setting 7.0, followed by 30 minutes of incubation. Ninety six cycles of sonication were preformed over 48 hours, with a 25µl aliquot transferred to a fresh NBH preparation for serial amplification. Following three rounds of amplification, samples were evaluated by western blotting, as described above, for the presence of PrP^{CWD}.

RESULTS

To investigate whether urine may play a role in natural CWD transmission, and to confirm the presence of PrP^{CWD} in saliva, pooled and concentrated urine or saliva from five terminally infected CWD+ deer was inoculated into two groups of *tg1536* mice. Following inoculation, mice were monitored for clinical signs of prion infection and were euthanized when terminal disease was apparent. Central nervous system tissues were evaluated for PrP^{CWD} using western blotting (WB), immunohistochemistry (IHC) and, when negative by conventional assays, serial protein misfolding cyclic amplification (sPMCA).

In a group of 9 mice inoculated with lyophilized urine, 2 animals developed neurologic disease consistent with a TSE (Table 1.1), including ataxia, a slow, lumbering gait, and poor thrift, at 370 and 376 days post-inoculation (dpi). Eight out of 9 mice inoculated with prepared saliva likewise developed signs of TSE at 342+/-109 dpi. All mice in positive control groups, inoculated with CWD+ brain spiked into either urine or saliva, developed disease before 370 dpi (235+/-91dpi), while none of the mice in either negative control group, inoculated with either urine or saliva spiked with negative brain homogenate, demonstrated clinical evidence of a TSE after >640 days.

Inoculum	WB+	IHC+	PMCA+	Incubation Period
(+) Control	18/18	18/18	NA	235+/-91d
Urine	2/9	2/9	1/7	373+/-3d
Saliva	8/9	8/9	0/1	342+/-109d
(-) Control	0/18	0/18	0/18	>640d

Table 1.1: Western Blot (WB), immunohistochemistry (IHC), and protein-misfolding cyclic amplification results and incubation periods of Tg[CerPrP] mouse bioassay. Numerators indicate the number of animals testing positive by a particular assay, while denominators designate the total number tested. PMCA analysis was reserved for mice testing negative by traditional assays. Incubation periods indicate the survival times in days post inoculation +/- one standard deviation.

Mouse bioassay of saliva and urine reveal the presence of infectious CWD prions:

Brains of all mice demonstrating terminal neurologic disease, including 2 of 9 inoculated with urine and 8 of 9 inoculated with saliva, had evidence of protease-resistant prion protein by both WB and IHC. PrP^{CWD} was absent by both WB and IHC in mice not displaying clinical disease, including those in negative control groups.

Immunohistochemistry demonstrated widely distributed, florid PrP^{CWD} plaques, with no apparent relationship between deposition pattern, lesion severity, and source inoculum (Figure 1.1). In cases with the least severe pathology, however, cortical lesions predominated; with increasing neuropathology, lesions were further distributed within the hippocampus, midbrain, and cerebellum.

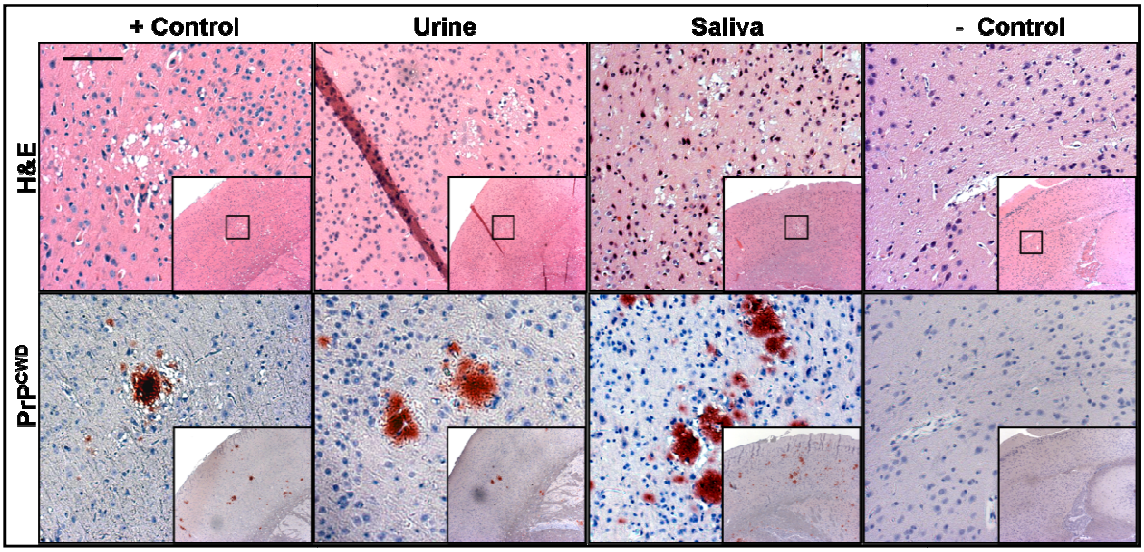

Figure 1.1: Spongiform degeneration and PrP^{CWD} identified by histopathology and immunohistochemistry. Vacuolated neurons and spongiform degeneration of the neuropil characteristic of a TSE is evident on H&E staining, with the colocalization of PrP^{CWD} specific immunostaining of florid plaques in the cortices of mice inoculated with positive control inoculum and concentrated urine and saliva from CWD-infected cervids. Negative control mice showed no evidence of spongiform degeneration or PrP^{CWD} immunostaining. HRP-conjugated BAR-224 was used as a primary antibody. (Measure bar, 50 µm)

In western blotting, PrP^{CWD} proteinase K-resistant glycoforms spanned 21-27 kD. In all cases, the dominant PrP^{CWD} glycoform was the di-glycosylated band, followed by mono- and non-glycosylated isoforms (Figure 1.2).

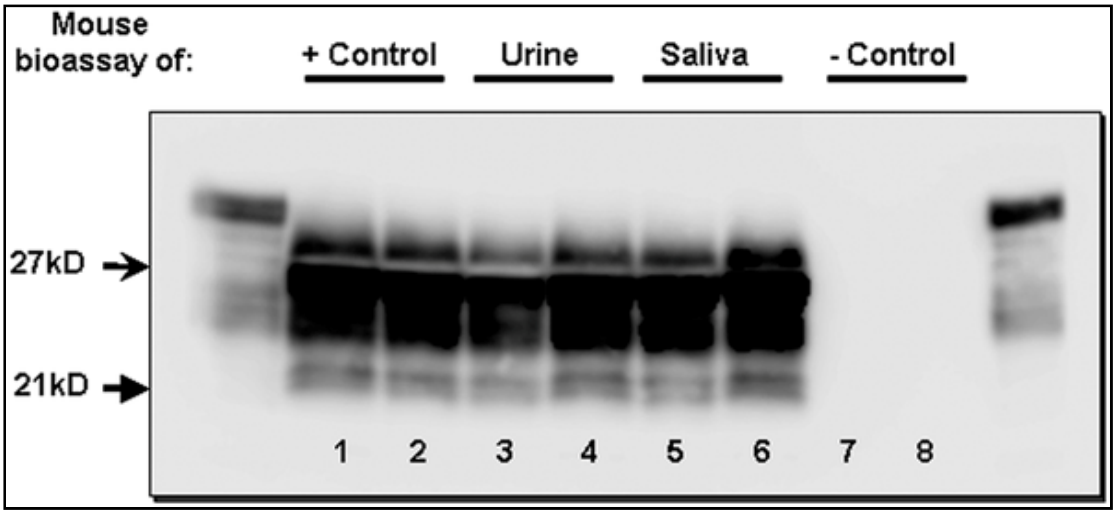

Figure 1.2: Western Blot detection of PrP^{CWD} in urine and saliva-inoculated mice. Western blotting analysis of control and test mice, demonstrating PrP^{CWD} in positive control mice (lanes 1 and 2), as well as urine (lanes 3 and 4) and saliva (lanes 5 and 6) inoculated mice. Protease-resistant prions were not detected in negative control mice (lanes 7 and 8). Flanking lanes represent undigested PrP^{C}.

Cervid urine donors display evidence of renal pathology on histological evaluation:

To identify potential pathological mechanisms for prionuria, histopathologic examination of donor renal tissues was also performed. Microscopic evaluation of H&E stained kidney sections from each of the donor deer revealed minimal histologic disease in 4 of the 5 animals. Lesions in these animals were characterized by the combination of minimal proliferative glomerular disease and mild interstitial fibrosis and lymphocytic inflammation (Figure 1.3A and B). In these animals, there was no appreciable histologic evidence of proteinuria or pyelonephritis. In the fifth animal, more significant renal pathology was detected. In this animal, there was a combination of mild, chronic, lymphocytic glomerulonephritis, which was similar to the previous 4 animals, and a moderately severe, chronic, lymphocytic interstitial nephritis with light microscopic evidence of renal protein loss ("tubular proteinosis"). (Figures 1.3C and D)

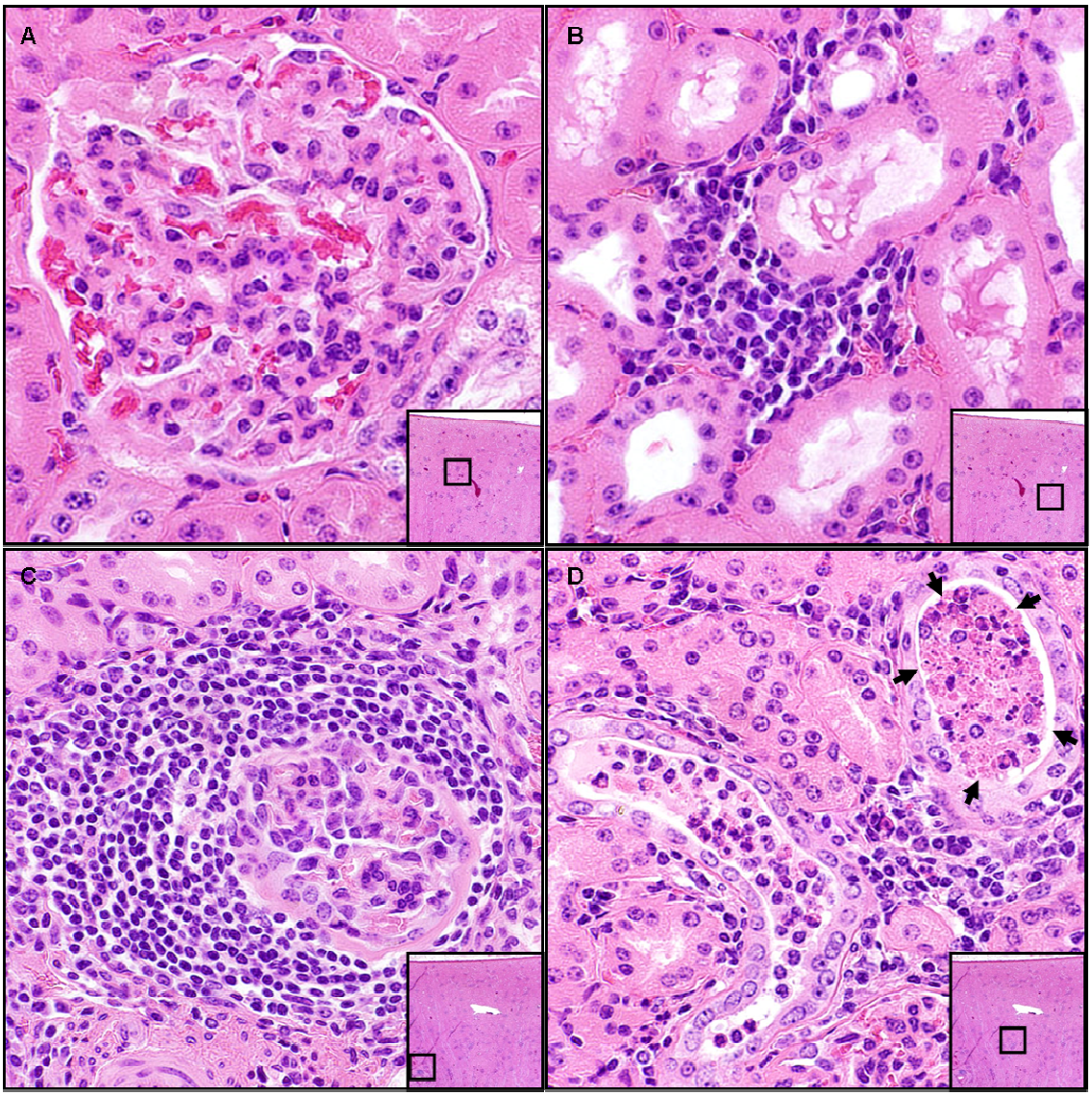

Figure 1.3: Histopathologic evaluation of renal tissues from donor cervids. (A) Minimal, chronic and proliferative glomerular disease and (B) mild interstitial fibrosis and lymphocytic infiltration were observed in 4 out of 5 donor deer. The remaining deer showed evidence of mild lymphocytic glomerulonephritis (C) as well as "tubular proteinosis." (D, arrows)

Serial PMCA may amplify PrP^{CWD} in conventional-test negative cervid urine samples and exposed mice:

Concentrated samples used for mouse inoculation were assayed for PrP^{CWD} by serial PMCA (sPMCA) over three rounds of amplification. In our experience, three

rounds of amplification permits an approximate 4000-fold increase in sensitivity as compared to traditional western blotting detection, while avoiding both cross-contamination and generation of any spontaneously formed protease-resistant PrP, thereby maintaining 100% specificity (Kurt, Perrott et al. 2007). In three independent experiments, PrP^{CWD} was identified in lyophilized urine homogenate from CWD+ deer. (Figure 1.4A) PrP^{CWD} was also found in both positive control inocula after the initial round of amplification, while PrP^{CWD} was not detected in either saliva or negative control preparations.

To increase detection sensitivity in bioassay experiments, brains from all mice that tested negative for PrP^{CWD} by WB and IHC, including negative controls, were re-evaluated by sPMCA. The brain from one WB- and IHC-negative mouse that had been inoculated with urine from a CWD+ deer and expired at 582dpi amplified PrP^{CWD} in three independent PMCA experiments (Figure 1.4B). No mice in either of the negative control groups were positive using this assay.

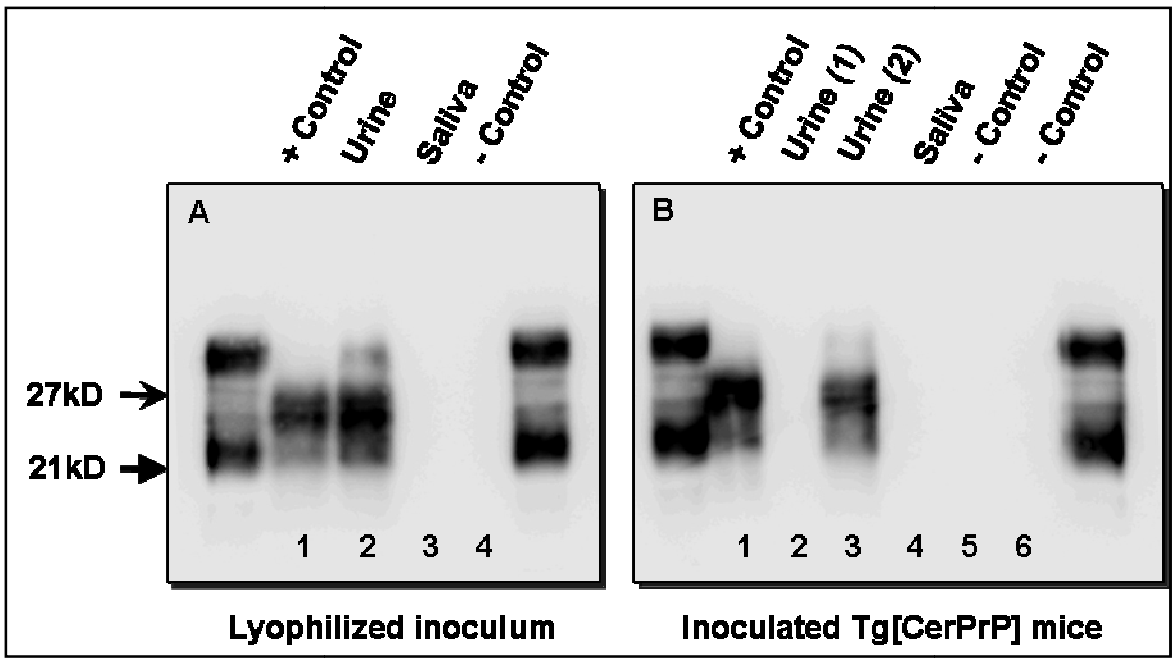

Figure 1.4. Serial PMCA amplification of PrP^{CWD} in concentrated deer urine and in the brains of urine-inoculated mice. A) PrP^{CWD} was detectable by serial PMCA (sPMCA) in control and urine inocula (lanes 1 and 2, respectively), while PrP^{CWD} could not be identified in saliva and negative control inocula (lanes 3 and 4, respectively) after 3 rounds of amplification. B) Three rounds of sPMCA also amplified PrP^{CWD} in the brains of CWD-infected mice, including positive-control inoculated mice and a single mouse inoculated with lyophilized urine (lanes 1 and 3, respectively). PrP^{CWD} was not amplified in mice inoculated with negative control material (lanes 5 and 6) or in other mice inoculated with either urine (lane 2) or saliva (lane 4) from CWD+ deer. All flanking lanes represent undigested PrP^{C}.

DISCUSSION

The salient feature of chronic wasting disease is its facile transmission among its host species. Until recently, little was known regarding the mechanisms of this efficient transmissibility, however, previous research has demonstrated infectious prions in the saliva and blood of infected deer (Mathiason, Powers et al. 2006). By using intracerebral inoculation of concentrated urine in cervid PrP transgenic mice, we report the presence of infectious prions in urine from CWD-infected cervids, and confirm the phenomenon of prionsialia in these animals. The identification of CWD prions in bodily fluids described in the current report could portend infectivity in secretions and excretions in other prion diseases.

In contrast to the data presented here, oral inoculation of urine in cervid bioassays was unable to identify infectious prions in the urine of CWD+ deer (Mathiason, Powers et al. 2006). This result could have been due to necessarily limited observation period possible in those studies (19 months), or variations in source and recipient genotype (Hamir, Gidlewski et al. 2006; O'Rourke, Spraker et al. 2007), route of inoculation (Hamir, Kunkle et al. 2005), or the sensitivity of traditional PrP^{CWD} detection assays (Safar, Geschwind et al. 2005; Grassi, Maillet et al. 2008). The mule deer providing inoculum pools in prior studies were of an unreported genotype; the majority of the recipient deer were homozygous for glycine at residue 96, although a single animal was heterozygous; sharing both G96 and S96 alleles (Mathiason, Powers et al. 2006). Likewise, the inocula used in the present study were pooled from sources heterogeneous at codon 96 of the cervid prion gene. Transgenic mice used in bioassay studies, on the

other hand, were uniformly homogenous for a glycine residue at this position (Browning, Mason et al. 2004), a polymorphism which is reported to be overrepresented in CWD-infected deer (Johnson, Johnson et al. 2003). As a result, it is possible that the genotypic background of either source or subject animals may have been a factor in susceptibility, though at present, we are unable to draw any concrete conclusions regarding this relationship. While mouse genotype may have played a role in the outcome, it is also probable that cervid PrP transgenic mouse bioassay simply represents a more sensitive detection system for prions in excreta. Intracranial inoculation, reportedly a more sensitive route of prion exposure (Hamir, Kunkle et al. 2005; Hamir, Kunkle et al. 2008), is more easily performed in mouse bioassay, a model which also permits extended incubation periods and inclusion of a greater number of test animals.

While my findings point to urine as an additional vehicle for CWD transmission, only 2 of 9 inoculated *tg1536* mice were confirmed WB/IHC-positive for prion infection, with a third PrP^{CWD}+ animal later identified by PMCA. This contrasts with 8 of 9 positive mice receiving saliva and infers a much lower concentration of prion infectivity in urine. The wide range of survival times in inoculated mice suggests relatively low levels of infectious prions and/or uneven distribution of infectious PrP moieties in the inocula (Kariv-Inbal, Ben-Hur et al. 2006). Differing [CerPrP] zygosity in *tg1536* mice (homozygous vs. hemizygous) may also have played a role in this variation (Browning, Mason et al. 2004).

Using sPMCA, PrP^{CWD} was repeatedly identified in test urine and spiked urine and saliva used as positive control, but was not detected in test saliva after three rounds of amplification. The reasons for our inability to identify PrP^{CWD} in saliva – given the definitive bioassay findings – remain unknown, and we propose the presence of as-yet unidentified inhibitors such as mucin or salivary proteases which are thought to negatively affect other *in vitro* assays (Archibald and Cole 1990; Ochert, Boulter et al. 1994).

The finding of PrP^{CWD} in urine and saliva calls for the identification of the pathological processes and cellular associations of the prion protein involved in shedding. Previous studies have related renal pathology to prionuria (Seeger, Heikenwalder et al. 2005; Siso, Gonzalez et al. 2006), a finding which corresponds to the identification of mild to moderate nephritis in those deer providing samples for the current study. It is plausible that renal pathology contributed to prionuria in each of these animals; as samples were pooled, however, we cannot identify specific animals in which it may have been occurring, nor can we accurately estimate the relative level of prionuria occurring in each donor as ultrastructural studies were not performed (Nacar, Karabay et al. 2008). While we have not yet identified pathologic prions in renal source tissues [Unpublished data], protease-resistant PrP^{CWD} has been identified by immunostaining in renal tissue of prion-infected deer (Hamir, Kunkle et al. 2006), sheep (Ligios, Cancedda et al. 2007), hamsters and most intriguingly humans (Fournier, Escaig-Haye et al. 1998), foreshadowing the potential for prionuria in other transmissible spongiform

encephalopathies. We continue to examine tissues from CWD+ deer in an effort to determine the pathogenesis and kinetics of CWD prion excretion and shedding.

Evidence for excretion and shedding of infectious prions is also accumulating in the scrapie system. PrP^{C}-converting activity has been identified by sPMCA in the urine of scrapie-infected sheep, hamsters and mice (Kariv-Inbal, Ben-Hur et al. 2006; Murayama, Yoshioka et al. 2007; Andrievskaia, Algire et al. 2008; Gonzalez-Romero, Barria et al. 2008). Prion infectivity has also been demonstrated in the feces of hamsters orally infected with scrapie (Safar, Lessard et al. 2008). Other studies point to infectious prions in the milk of scrapie-infected ewes (Gresham 2008; Konold, Moore et al. 2008). As noted above, it remains unknown whether other prion diseases (e.g. Kuru, BSE, CJD, TME) may be transmitted by bodily fluids or excreta other than blood. Additional studies examining feces, milk, and other body fluids are therefore necessary in CWD and other prion diseases, studies currently underway in our laboratory.

As CWD transmission may model communicability of other TSE's, the transmissible nature of prion diseases *may* serve as a model for other protein-misfolding diseases. For example, feces, but not urine, from both mice and cheetahs affected with systemic amyloidosis A (SAA) was recently shown to induce SAA in a mouse model, although negative controls were not available in those studies (Zhang, Une et al. 2008). In light of the prionuria detected in CWD and in models of scrapie, further investigations of infectivity in body fluids in other protein folding diseases may be warranted in the event that prion diseases are not the only infectious proteinopathies.

In summary, we have confirmed prionsialia in CWD-affected deer by bioassay in cervidized mice and demonstrate for the first time infectious prions in the urine of these cervids by both bioassay and sPMCA. We are currently evaluating urine and saliva from individual animals in hopes of identifying predisposing factors, such as genotypic background and underlying pathology, which may contribute to prionuria and prionsialia. Concurrently, we have begun to explore the tissue origins and protease sensitivity of the infectious prions as well as the onset and duration of shedding in these bodily fluids.

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Chapter 2:

Detection of Subclinical CWD Infection in Conventional Test-Negative Deer Long After Exposure to Urine and Feces

ABSTRACT

Chronic wasting disease (CWD) of cervids is a prion disease distinguished by its high level of transmissibility, wherein bodily fluids and excretions are thought to play an important role. Using cervid bioassay and established CWD detection methods, experiments have previously identified infectious prions in saliva and blood but not urine or feces of CWD+ donors. More recently, our research has identified very low concentrations of CWD prions in urine of deer by cervid PrP transgenic (Tg[CerPrP]) mouse bioassay and serial protein misfolding cyclic amplification (sPMCA). This finding led us to examine further those initial cervid bioassay experiments using sPMCA. We sought to determine whether deer previously exposed orally to urine and feces from CWD+ sources, while conventional test-negative, may actually be harboring very low level CWD infection not evident in the 19 month observation period in the previous studies. We further attempted to trace the centripetal spread of PrP^{CWD} in these animals from the gastrointestinal tract to the central nervous system in these animals. Obex, vagal nerve, intermediolateral spinal cord segments, sections of ileum, and a wide range of lymphoreticular system (LRS) tissues from conventional test-negative deer were reanalyzed for CWD prions by sPMCA and cervid transgenic mouse bioassay in parallel with appropriate tissue-matched positive and negative controls. PrP^{CWD} was detected in the tissues of orally exposed deer by both sPMCA and Tg[CerPrP] mouse bioassay; each assay revealed very low levels of CWD prions previously undetectable by western blot, ELISA, or IHC. Serial PMCA analysis of individual tissues identified that obex alone was positive in urine/feces exposed deer. PrP^{CWD} was amplified from both LRS and neural tissues of positive control deer but not from the same tissues of negative control

deer. Detection of subclinical infection in deer orally exposed to urine and feces, (1) suggests that a prolonged subclinical state can exist such that observation periods in excess of two years may be needed to detect CWD infection, and (2) illustrates the sensitive and specific application of sPMCA in the diagnosis of low-level prion infection. Based on these results, it is possible that low doses of prions, such as via oral exposure to urine and saliva of CWD-infected deer, bypass significant amplification in the LRS, perhaps utilizing a neural conduit between the alimentary tract and central nervous system, as has been demonstrated in some other prion diseases.

BACKGROUND

Chronic wasting disease (CWD) is an efficiently transmitted prion disease of cervids (e.g. deer, elk, and moose), and is the only known prion disease affecting free-ranging, non-domestic animals. The origins of CWD are uncertain, but the disease has been present in wild cervid populations of northern Colorado and southern Wyoming for at least 40 years (Williams and Young 1980; Williams and Young 1982). Since its discovery, CWD has been identified in captive and free-ranging cervids in 15 states, 2 Canadian provinces, and Korea (Sigurdson 2008). As surveillance efforts have intensified, CWD has been detected in areas previously thought to be free of infection, including recent discoveries in West Virginia, New York, and Michigan (NYSDAM 2005; WVDNR 2005; MDNR 2008). The prevalence of CWD varies across North America, but can be as high as 30% in some areas of Colorado or in captive populations (Williams 2005; Keane, Barr et al. 2008).

Although the mechanisms of CWD transmission are incompletely understood, there is evidence inferring that infection is transmitted horizontally via saliva and urine (Mathiason, Powers et al. 2006; Mathiason, Hays et al. 2009), and may be acquired from the environment (Miller, Williams et al. 2004; Johnson, Phillips et al. 2006; Johnson, Pedersen et al. 2007). With the expanded recognition of the disease across the continental United States, it is also likely that substantial human exposure has occurred. However, because of an apparently strong species barrier (Raymond, Bossers et al. 2000; Kong, Huang et al. 2005) and the as yet incompletely understood natural routes and

kinetics of CWD transmission, the magnitude and consequence of this exposure remain speculative.

While infectious CWD prions have been detected in saliva, blood, urine, and feces (i.e. prionsialia, prionemia, prionuria, and prionorrhea) conventional CWD diagnostic assays (e.g. western blotting, immunohistochemistry, and enzyme-linked immunosorbent assay) have been unable to identify PrP^{CWD} in these materials (Mathiason, Powers et al. 2006; Haley, Seelig et al. 2009; Mathiason, Hays et al. 2009; Tamguney, Miller et al. 2009). In the same vein, the early and antemortem identification of subclinically infected individuals requires demonstration of PrP^{CWD} in biopsied lymphoreticular system (LRS) tissues (Wild, Spraker et al. 2002; O'Rourke, Zhuang et al. 2003; Spraker, Gidlewski et al. 2006; Wolfe, Spraker et al. 2007). Improving the sensitivity of low level of CWD prion detection in excreta or at early sites of accumulation may allow for earlier antemortem diagnosis and a stronger estimate of prevalence (Hill and Collinge 2003; Hill and Collinge 2003; Castilla, Gutierrez-Adan et al. 2004; Spraker, Balachandran et al. 2004; Haley, Seelig et al. 2009), an approach which could also aid in detection of human transmissible spongiform encephalopathies (TSE's).

Apart from inoculation of susceptible hosts (bioassay), only *in vitro* amplification by serial protein misfolding amplification (sPMCA) (Saborio, Permanne et al. 2001; Soto, Saborio et al. 2002; Kurt, Perrott et al. 2007; Haley, Seelig et al. 2009) offers the potential for comparable sensitivity. In these experiments, we have employed both of the

latter methods to demonstrate low levels of infectious prions in clinically normal, conventional assay-negative white-tailed deer orally exposed 19 months previously to urine and feces from CWD+ deer.

MATERIALS and METHODS

Infected cervids:

Five white-tailed deer (*Odocoileus virginianus*) were orally inoculated with urine (50ml total volume) and feces (50g total volume) from CWD+ donor deer. The deer were monitored for 19 months post inoculation (pi), during which time they remained asymptomatic. At 19 months pi, due to limitations in holding space imposed by other ongoing studies, the animals were euthanized, necropsied, and brain and lymphoid tissues examined for PrP^{CWD} by western blotting (WB) and immunohistochemistry (IHC) (Mathiason, Powers et al. 2006). The sources of the urine and feces used for inoculation were three terminally-ill mule deer (*Odocoileus hemionus*) of unknown PrP genotype. Three of the inoculated deer were homozygous for glycine (i.e. G/G) at cervid PrP position 96 (Deer #'s 134, 141, and 150), while two were heterozygous at that location (Deer #'s 111 and 124), with alleles encoding for both glycine and serine (G/S). Tissues from all five animals were negative for PrP^{CWD} by WB and IHC and the animals were thus considered CWD-negative. The same tissue sets were collected from positive and negative control animals, including a deer inoculated intracranially (IC) with CWD+ brain (deer #106, G/S at position 96), two deer inoculated *per os* (PO) with saliva from CWD+ deer (deer #113 and 122, both G/G at position 96) and three deer inoculated by the IC and PO routes with brain homogenate from a CWD-naive deer (deer # 103 and 123, both G/G at position 96 and deer #4488 – G/S at this loci). Each deer was necropsied using fresh necropsy instruments, and all tissue samples were frozen at -70° C until use. All animals were maintained in accord with Colorado State University IACUC guidelines.

Study samples and preparation:

Obex, vagal nerve, intermediolateral spinal cord segments, sections of ileum, and a number of lymphoid tissues, including tonsil and retropharyngeal, mesenteric, mediastinal, and ileocecocolic lymph nodes, were collected at necropsy and frozen at -70°C. In initial sPMCA and bioassay experiments, obex and retropharyngeal lymph node (RLN) tissues from individual animals were pooled, as both these tissues have proven sensitive in the identification of PrP^{CWD} at various stages of infection (Sigurdson, Williams et al. 1999; Hibler, Wilson et al. 2003; Spraker, Balachandran et al. 2004; Fox, Jewell et al. 2006; Race, Meade-White et al. 2007; Keane, Barr et al. 2008). In later serial PMCA (sPMCA) experiments, specific neural and LRS tissues were analyzed individually. In each case, a fifty milligram section of each tissue was washed twice in phosphate-buffered saline (PBS), then homogenized and prepared as a 1% solution (w/v) in PBS using a FastPrep™ tissue homogenizer for 40s at power setting 6.5. All tissues were prepared in individual microcentrifuge tubes and homogenized in parallel in the same machine concurrent with controls.

Cervid PrP transgenic mice:

Tg[CerPrP] line 5037 (*tg5037*) mice were generated in the Telling laboratory at the University of Kentucky (Angers, Seward et al. 2009). These mice express, both centrally and peripherally, an allelic variant of the prion gene possessed by Rocky Mountain Elk (*Cervus elaphus*), coding for glutamic acid at position 226 of the cervid prion protein. All mice were screened at weaning for the presence of the cervid *PRNP* transgene by conventional and real-time PCR. Mice testing negative for PrP^{CWD} at the

completion of bioassay studies were rescreened to confirm the presence of cervid transgene. Mice were inoculated and maintained in accord with Colorado State University IACUC guidelines.

Serial Protein Misfolding Cyclic Amplification (sPMCA):

Tissue homogenates from CWD-exposed deer, negative by WB, IHC, and ELISA, were assayed for PrP^{CWD} by sPMCA. In initial experiments, CWD+ obex/RLN homogenate from a white-tailed deer IC-inoculated with CWD+ brain (deer #106) was used as a positive control, while tissue preparations from two sham-inoculated deer (#103 and 123) and untreated *tg5037* mice were used as negative controls. In later experiments, a tissue set from a deer orally inoculated with CWD+ brain (deer #148) was selected for use as positive controls; negative control animals remained unchanged. All test samples were prepared in parallel with tissue-matched positive and negative controls as a 1% homogenate in PBS as described above and subsequently spiked into normal brain homogenate for amplification as described previously (Kurt, Perrott et al. 2007; Haley, Seelig et al. 2009; Kurt, Telling et al. 2009). Normal brain homogenate (NBH), the substrate for prion conversion *in vitro*, was prepared from *tg5037* mice in a room that had not previously been used for prion research. Following euthanasia and perfusion with 5mM EDTA in phosphate-buffered saline (PBS), whole brain was collected from naïve *tg5037* mice and placed on ice. Brain homogenates were prepared as a 10% (w/v) solution in PMCA buffer (1% triton-X 100 [v/v], 5mM EDTA, and 150mM NaCl in PBS adjusted to a pH of 7.2) with the addition of Complete Protease Inhibitors (Roche Pharmaceuticals, Indianapolis, IN) using a dounce homogenizer. Homogenates were

then centrifuged for 1 minute at 2000rpm and the supernatant frozen in single-experiment aliquots at –70ºC in a "prion-free" room until use in PMCA. Fifteen µl of test or control tissue homogenate was added to 45µl of NBH and assayed, in parallel and in adjacent wells of a 96-well plate (USA Scientific, Ocala, FL); along with normal brain homogenate prepared from unexposed *tg5037* mice as additional, unseeded negative controls. Plates were then sonicated using an ultrasonic processor (Misonix, Farmingdale, NY) and incubated at 37ºC. Sonication parameters were set at 40s bursts at power level 7.0, followed by 30 minutes of incubation. Ninety six cycles of sonication were performed over 48 hours, with a 10µl aliquot transferred to 50µl of fresh NBH for serial amplification. Following three rounds of amplification, samples were evaluated by western blotting, as described below, for the presence of PrP^{CWD}. Brain homogenates from all mice testing negative for PrP^{CWD} in bioassay experiments were likewise analyzed to increase detection sensitivity. In analysis of individual cervid tissue samples, occasional differences were noted in the round in which amplification was initially observed. For this reason, we tallied the number of successive positive rounds for each sample in each repetition for semi-quantitative analysis. Over the course of sPMCA experiments, multiple NBH preparations were used, and each test or control sample was evaluated at least three times for repeated verification of results.

Mouse bioassays:

Four groups of *tg5037* mice (n=8/group) were anesthetized with ketamine and xylazine and inoculated intracerebrally into the left parietal lobe with 30µl of 1% obex/RLN homogenate. Positive control mice were inoculated with obex and RLN from

a deer IC-inoculated with CWD+ brain (deer #106), while a single negative control group was inoculated with combined obex and RLN homogenates from two sham-inoculated deer (deer #103 and 123). Obex and RLN homogenates from two deer orally inoculated with urine and feces (deer #134 and 150) were selected for bioassay experiments based on their apparent amplification ability in sPMCA experiments (below). Incubation time was defined as the number of days from inoculation to the onset of clinical signs of transmissible spongiform encephalopathy (TSE), as previously described (Carlson, Kingsbury et al. 1986). Animals were euthanized when either signs of clinical TSE or distress were evident. Brain harvested at necropsy was divided longitudinally, with one hemi-section prepared for evaluation by western blotting and sPMCA and the remaining hemi-section fixed in 10% neutral-buffered formalin for immunohistochemical analysis

Western blotting (WB):

Brain hemi-sections for WB and sPMCA were initially prepared as a 10% (w/v) solution in PMCA buffer. Eleven µl of sample homogenate were mixed with 7µl of sample buffer (0.1% [v/v] triton-X 100 and 4% [w/v] SDS in PBS) and digested with 2µl proteinase-K at 500µg/ml (final concentration: 50µg/ml) for 20' at 37ºC followed by 10' at 45ºC. Seven µl of 4X running buffer were then added to the sample, followed by denaturation for 5' at 95ºC. Twenty µl of this preparation were run on a pre-cast 12% SDS-PAGE gel (Invitrogen) in a Bio-Rad electrophoresis apparatus for 2 hours at 120mV. Samples were then transferred to a PVDF membrane (Millipore) for 1 hour at 110mV in a Bio-Rad transfer apparatus. PVDF membranes were subsequently blocked for 1 hour in 5%(w/v) powdered milk in 0.2% Tween-20 in tris-buffered saline (TBST),

followed by application of the primary antibody, BAR224-HRP, diluted 1:20,000 in TBST with 5% powdered milk, for 1 hour. Following washing, immunoreactivity was detected using an enhanced chemiluminescent detection system (ECL-plus, Amersham Biosciences) in a LAS 3000 imaging system. (Fuji Photo Film, Fuji Inc, Valhalla, NY)

Neuropathology and immunohistochemistry (IHC):

Brain hemi-sections were fixed in formalin overnight, treated with 88% formic acid for one hour, washed in tap water and then stored in 60% ethanol prior to paraffinization. Paraffin-embedded tissue sections (6µm) were mounted onto positively charged glass slides, deparaffinized, and rehydrated through graded ethanol. To enhance detection, tissues were subjected to Heat Induced Epitope Retrieval (HIER) using an automated antigen-retrieval system (Retriever™) and a proprietary buffer solution (DakoCytomation Target Retrieval Solution, DAKO, Hamburg, Germany). Tissues were then stained with an automated immunostainer, using polyclonal PrP antibody R-505 as the primary antibody (a gift from Dr. Jan Langeveld, Central Veterinary Institute of Wageningen University), The Netherlands) at a 1:500 final dilution, followed by secondary application of a universal anti-rabbit polyclonal antibody conjugated to horseradish peroxidase (HRP). Detection was completed using HRP-mediated hydrogen peroxide immunostaining (AEC+, DAKO), with hematoxylin as a counterstain.

RESULTS

To evaluate CWD-exposed, yet IHC-, and WB-negative, deer for subclinical CWD infection, we pooled obex and retropharyngeal lymph node (RLN) tissues in an effort to enhance detection sensitivity. Pooled tissues from individual animals were initially analyzed via serial PMCA (sPMCA) with results subsequently confirmed in two of these individuals using bioassay in cervidized *tg5037* mice. Serial PMCA was then used to analyze individual nervous and LRS tissues to estimate the mechanism of CNS invasion in animals orally exposed to presumed low doses of CWD prions that may be present in urine and feces.

Serial PMCA amplification of PrP^{CWD} from tissues of deer orally exposed to urine and feces:

Obex and RLN tissues collected at necropsy from experimentally exposed deer were prepared as a 1% solution in PBS and subjected to three rounds of sPMCA as described. In our experience, three rounds of amplification permits up to 4000-fold increase in sensitivity as compared to traditional western blotting detection, while maintaining 100% specificity (Kurt, Perrott et al. 2007; Haley, Seelig et al. 2009). In three independent experiments, obex and RLN homogenates from individual deer that had been orally inoculated with urine and feces from a CWD+ source (deer #'s 111, 124, 134, 141 and 150) consistently amplified PrP^{CWD} by sPMCA. Positive control tissues from an IC-inoculated deer (deer #106) also successfully amplified PrP^{CWD} through each round, while concurrently run control tissue homogenates from CWD-negative brain-

inoculated deer (deer #'s 103 and 123) or naïve *tg5037* mice were negative by sPMCA. (Figure 2.1)

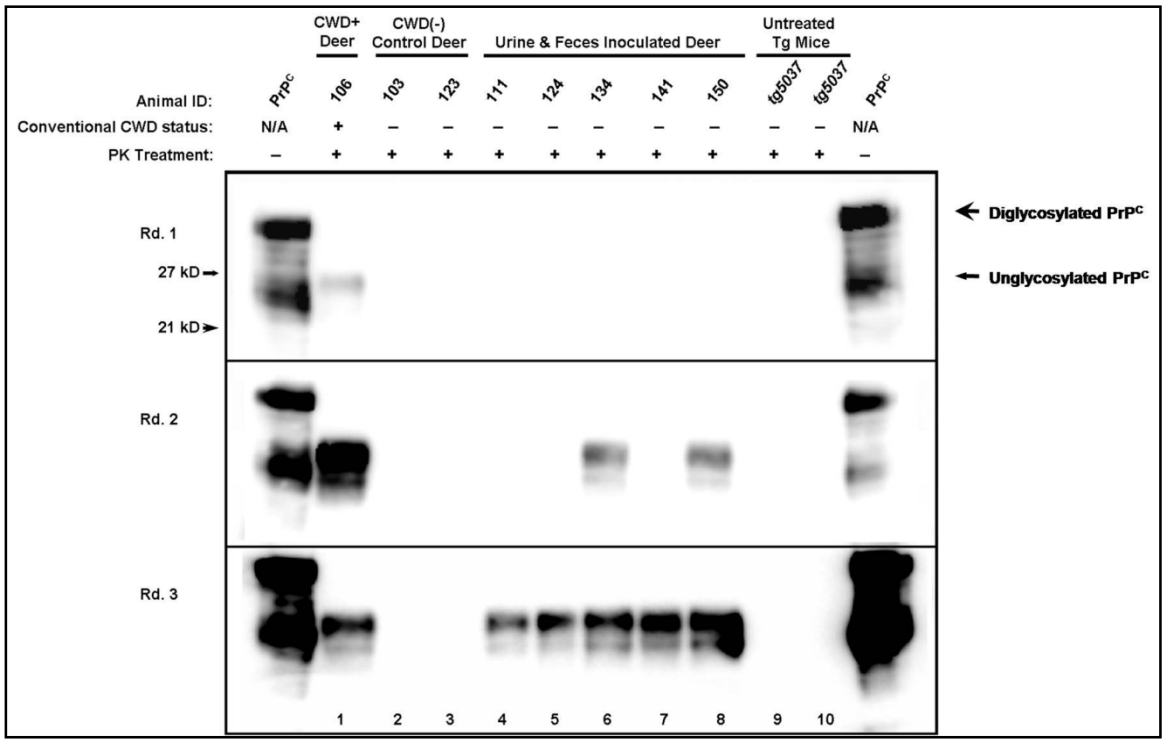

Figure 2.1: Serial PMCA amplification of CWD prions in WB and IHC negative deer. Conventionally negative tissues from deer orally exposed to urine and feces from CWD+ sources (Deer #'s 111, 124, 134, 141, and 150, lanes 4-8) amplified PrP^{CWD} after 2-3 rounds of PMCA, as did positive control tissues from deer #106 (lane 1). Tissue samples from two sham-inoculated deer (#103 and 123, lanes 2 and 3) and two untreated *tg5037* mice (lanes 9 and 10) failed to amplify PrP^{CWD} in three rounds of sPMCA.

Based on these findings, tissues from two white-tailed deer with the greatest apparent *in vitro* amplification ability (deer #134 and #150, Figure 2.1), along with tissue-matched controls, were selected for further evaluation by *tg5037* mouse bioassay. Groups of mice were inoculated with obex/RLN homogenates, monitored for clinical signs of prion infection, and euthanized when terminal disease was apparent. Brains

from inoculated mice were evaluated for PrP^{CWD} by WB, IHC and, when negative by these assays, also by sPMCA.

Authentic prion infectivity identified in tg5037 mouse bioassay:

In a group of 8 mice inoculated with obex/RLN homogenates from deer #134, 7 of the 8 mice developed clinical signs consistent with TSE, including progressive ataxia and weight loss, by 264 days post inoculation (dpi). WB and IHC confirmed the presence of PrP^{CWD} in each of these seven mice. The remaining mouse in this group died of intercurrent disease at 161 dpi. This mouse showed no evidence of clinical TSE and was negative for PrP^{CWD} by both IHC and WB (Figure 2.3, mouse #2-B). In a second group of mice, inoculated with tissues from deer #150, 8 of 8 mice developed similar clinical signs of TSE infection and were confirmed PrP^{CWD}-positive by WB and IHC by 272 dpi. All mice inoculated with brain and RLN homogenates from positive control deer #106 developed clinical TSE and were PrP^{CWD}-positive by 173 dpi, whereas negative control mice remained healthy until euthanasia at 340+ dpi, at which time they were also negative for PrP^{CWD} by WB and IHC. (Figure 2.2 and Table 2.1)

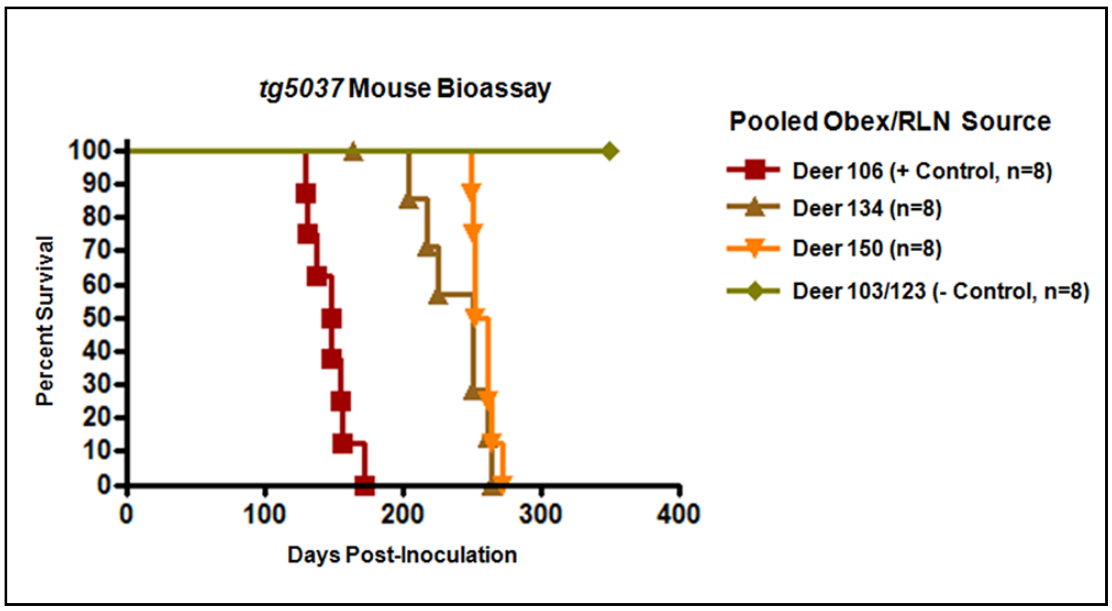

Figure 2.2: *Tg5037* mouse bioassay results. Kaplan-Meyer curve demonstrating prolonged incubation periods in mice inoculated with tissues from deer #134 and 150 as compared to mice inoculated with tissues from deer testing positive by conventional assays.

Inoculum	IHC +'s	WB +'s	sPMCA +'s	Incubation Period
106	8/8	8/8	N/A	148 (11)
134	7/8	7/8	1/1	238 (23)
150	8/8	8/8	N/A	259 (8)
103/123	0/8	0/8	0/8	340+

Table 2.1: Summary of immunohistochemistry (IHC), western blot (WB), and serial PMCA results and *Tg5037* mouse bioassay of combined obex/RLN homogenates. Mean incubation periods with standard deviations in parentheses; numerators indicate number of animals testing positive over total number tested. For PMCA, only those animals testing negative by IHC and WB were assayed. N/A: not assayed.

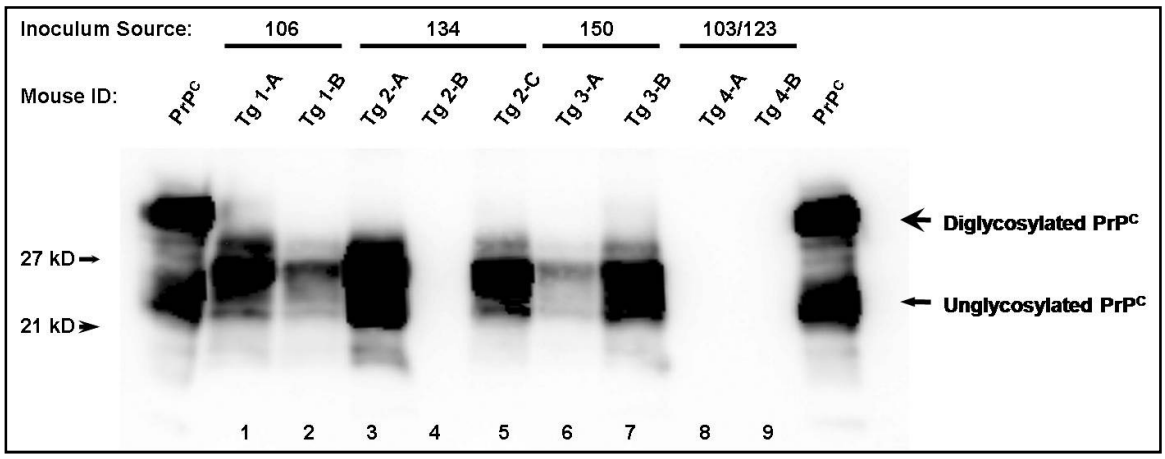

Figure 2.3: Western blot detection of PrP^{CWD} in mouse CNS tissues. Except for a single mouse (mouse Tg 2-B, lane 4), all mice inoculated with tissues from deer #134 and 150 succumbed to prion disease (lanes 3-7), as did mice inoculated with CWD+ deer #106 (lanes 1 and 2). Mice inoculated with tissues from sham-inoculated deer showed no evidence of PrP^{CWD} by western blot (lanes 8 and 9).

Biochemical confirmation of bioassay:

Western blot glycoform patterns were typical of CWD in Tg[CerPrP] *tg5037* mice, spanning 21-27 kD following proteinase-K digestion and dominated by a di-glycosylated PrP^{CWD} isoform. Representative WB's from each group are shown in Figure 3. Immunohistochemistry demonstrated a relatively narrow distribution of PrP^{CWD} within the hippocampus of affected mice, colocalizing with vacuolization and spongiform degeneration of the neuropil. There was no apparent relationship between deposition pattern, lesion intensity, and source inoculum. (Figure 2.4)

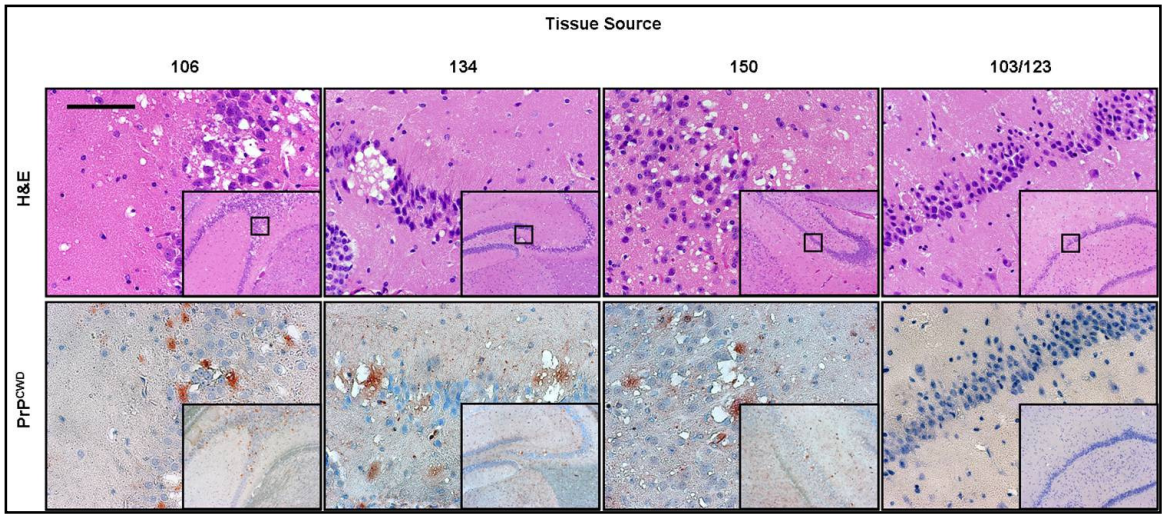

Figure 2.4: Spongiform degeneration and PrP^{CWD} in the hippocampus of inoculated mice. Vacuolated neurons and spongiform degeneration of the neuropil characteristic of TSE demonstrated by H&E staining and co-localization of PrP^{CWD} florid plaques in the hippocampus of mice inoculated with tissues from urine and feces exposed and positive control deer. Brains of mice inoculated with tissues from sham-inoculated deer showed no evidence of spongiform degeneration or PrP^{CWD} immunostaining. Anti-prion polyclonal antibody R-505 was used as the primary antibody. (Measure bar, 50 µm)

Serial PMCA identification of PrP^{CWD} in an asymptomatic mouse:

Negative control mice and a single test mouse expiring with intercurrent disease at 161dpi, all of which were found negative for PrP^{CWD} by WB and IHC, were further evaluated using sPMCA over three rounds of amplification. In three repeated sPMCA experiments, a subclinical prion infection was confirmed in the remaining mouse inoculated with tissue from deer #134 (mouse #Tg 2-B). None of the brains from mice inoculated with negative control tissues amplified PrP^{CWD}, while positive control mice amplified PrP^{CWD} successfully over three rounds. (Figure 2.5)

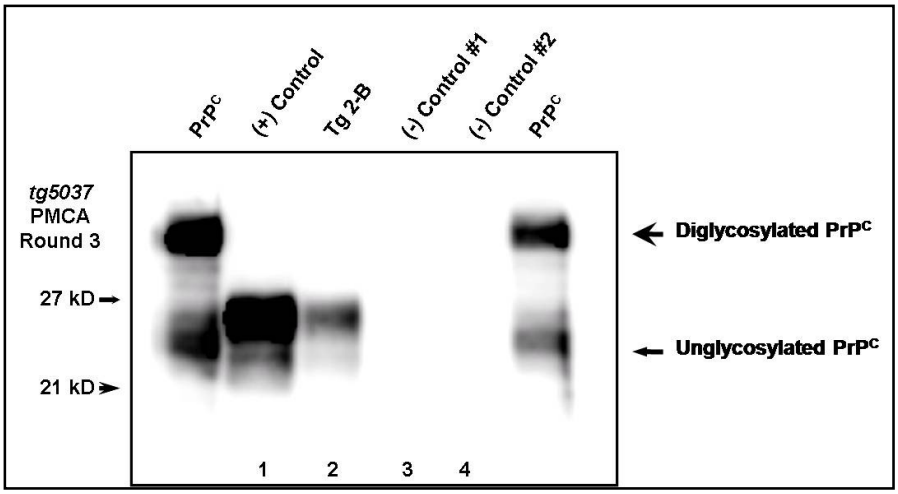

Figure 2.5. Serial PMCA detection of CWD prions in inoculated mice. Brain from a single *tg5037* mouse (mouse Tg 2-B) was WB and IHC negative yet amplified PrP^{CWD} after three rounds of sPMCA (lane 2), as did a positive control mouse (lane 1). Mice inoculated with tissues from sham-inoculated deer failed to amplify PrP^{CWD} (lanes 3 and 4).

Analysis of individual neural and lymphoid tissues by sPMCA:

To further examine the terminal distribution of CWD prions following oral exposure to presumed low concentrations of infectious prions in excreta, we analyzed individual tissue samples from control and test deer using sPMCA. Positive control tissues in these experiments included two deer inoculated PO with saliva from CWD+ deer (deer #113 and 122), while identical tissue sets were collected from negative control deer #103, 123, and 4488. By analyzing obex and retropharyngeal tissues individually, PrP^{CWD} amplification was found to occur exclusively in obex preparations from study animals. The levels of amplification observed were compared to serial dilutions of positive control tissue, and correlated to an approximately 20-40 fold dilution of 1% homogenate of deer #106 obex in NBH (data not shown). These results were surprising given that it has been reported that PrP^{CWD} is commonly found to accumulate in the

retropharyngeal lymph node, and other lymph nodes of the alimentary tract, prior to its appearance in the obex in experimental oral (Sigurdson, Williams et al. 1999) and natural CWD infections (Keane, Barr et al. 2008; Keane, Barr et al. 2008). We therefore sought to determine whether other lymphoid tissues were correspondingly bypassed in these deer.

In an attempt to understand if a strictly neural route of CNS invasion might have occurred, we performed sPMCA on the mesenteric, mediastinal, and ileocecocolic lymph nodes, tonsils, vagus nerve, intermediolateral spinal cord segments, and ileum from the above 5 urine/feces exposed deer and from positive and negative control deer. Surprisingly, while PrP^{CWD} was amplified from a terminal tonsil biopsy of a single study deer, neither vagus nerve, nor the retropharyngeal, mesenteric, mediastinal or ileocecocolic lymph nodes were positive for PrP^{CWD} by sPMCA in any of the deer orally exposed to urine and feces from CWD+ donors (Figure 2.6). As might be expected, protease-resistant prion protein was amplified from both neural and lymphoid tissues from the positive control deer, while no amplification was observed in corresponding tissues from negative control deer or multiple unseeded NBH controls examined concurrently and on the same plate as positive samples.

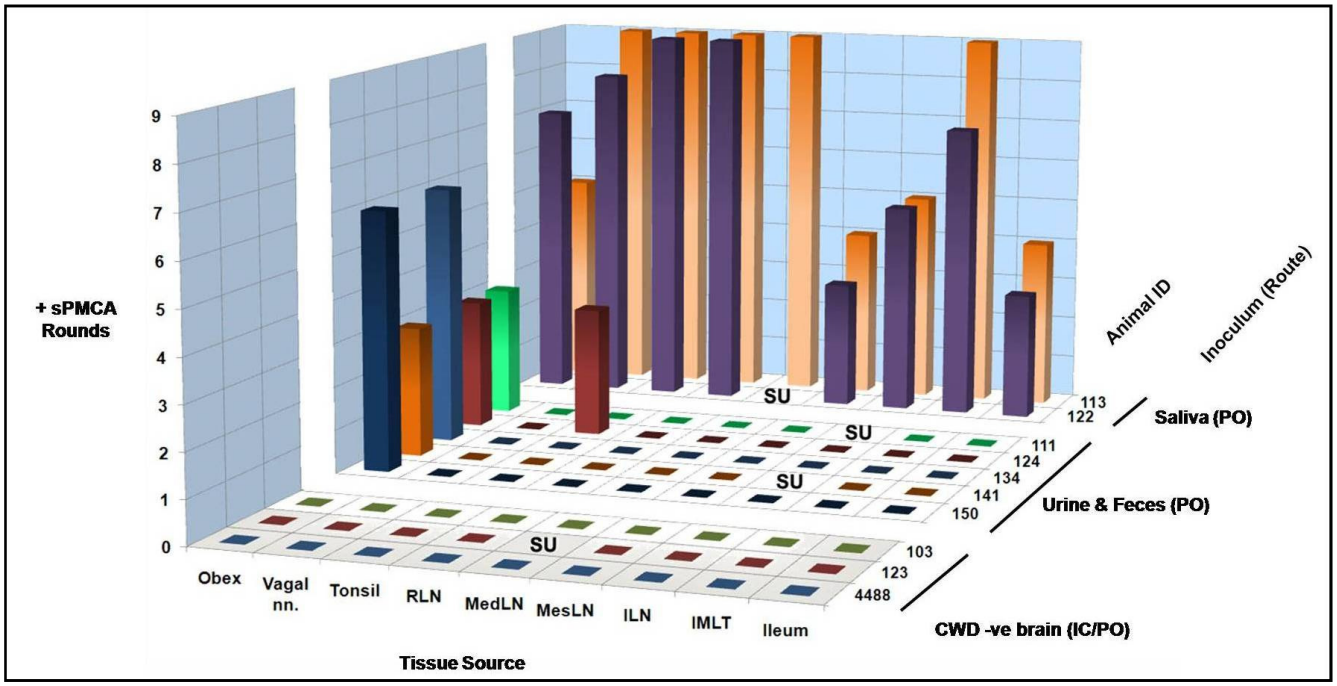

Figure 2.6: Serial PMCA detection or PrP^{CWD} of neural and lymphoid tissues of deer exposed orally to urine and feces of CWD-positive deer. For each tissue, the number of sPMCA rounds producing a positive result was tabulated for three independent experiments of three rounds each. Samples appearing as positive in round 2 continued to be positive in round 3, and thus over the course of three experiments, were positive a total of 6 out of 9 rounds (e.g. obex samples from deer 134, 150, and 148). Samples becoming positive in the first round were succeeded by positivity in rounds 2 and 3, and thus were positive in 9 out of 9 rounds (e.g. tonsil and RLN samples from deer 122). As would be expected, the greatest number of positive results was observed in the positive control tissues, with some variance among tissues and between animals. Deer orally exposed to urine and feces demonstrated PrP^{CWD} amplification almost exclusively from the obex; the terminal tonsil collection from a single animal also was positive. By contrast, negative control tissues failed to amplify PrP^{CWD}. RLN: retropharyngeal lymph node; MedLN: mediastinal lymph node; MesLN: mesenteric lymph node; ILN: ileocecocolic lymph node; IMLT: Intermediolateral spinal cord tract; SU: sample unavailable.

DISCUSSION

The salient feature of chronic wasting disease is its facile transmission among captive and free-ranging cervids. Prior research has demonstrated infectious prions in the saliva and blood using cervid bioassay (Mathiason, Powers et al. 2006; Mathiason, Hays et al. 2009). However, those experiments were unable to identify PrP^{CWD} in the tissues of deer orally exposed to combined urine and feces, even though our later studies employing Tg[CerPrP] mouse bioassay demonstrated very low levels of prion infectivity (surmised by long incubation/survival periods) in the urine of some CWD+ deer (Haley, Seelig et al. 2009). In the current study, the enhanced sensitivities of sPMCA and intracerebral inoculation of cervid PrP-*tg5037* mice have led us to conclude that at 19 months post inoculation, low levels of amplifiable and infectious CWD prions were in fact present in the brains of the exposed, yet asymptomatic and conventional assay negative, deer from our original oral bioassay studies (Mathiason, Powers et al. 2006; Mathiason, Hays et al. 2009), thereby inferring that low levels of prion infectivity were present in the urine/feces inocula. Whether the apparent low concentrations of prions amplifiable from the obex of the urine/feces recipients could represent a persistent non-pathogenic prion carrier state, or rather (perhaps more likely) indicates that an observation period far exceeding 19 months would be required to reveal ultimately pathogenic prion infections in these animals, remains undetermined.

Because the initial bioassay deer were inoculated with both urine and feces, our present findings cannot identify the specific excreta involved in CWD transmission. It seems likely, however, that both excreta may play an important role in the horizontal

transmission of CWD, given the later demonstration of prion infectivity in urine (Haley, Seelig et al. 2009) and feces (Tamguney, Miller et al. 2009) of CWD+ deer using transgenic mouse bioassay. Further experiments, employing cervid bioassay of separated excreta and sPMCA are underway to better answer this question.

In the initial sPMCA and bioassay experiments, to maximize use of available *tg5037* mice, we analyzed homogenates of combined neural (obex) and lymphoreticular (LRS) tissues for the CWD prion protein. Information on the pathogenesis of CWD indicates that, following oral exposure, PrP^{CWD} is first detectable in lymphatic tissues draining the alimentary tract, especially retropharyngeal lymph node and Peyer's patches (Sigurdson, Williams et al. 1999; Sigurdson, Spraker et al. 2001; Fox, Jewell et al. 2006; Keane, Barr et al. 2008; Keane, Barr et al. 2008). Thereafter, it is assumed that neural transport may be occur through anterograde ascension via myenteric or LRS sympathetic and parasympathetic neural networks to the central nervous system (Sigurdson, Spraker et al. 2001), paralleling the pathogenesis of sheep scrapie (Beekes and McBride 2007; van Keulen, Bossers et al. 2008). Thus the earliest and most prominent CNS PrP^{CWD} accumulation is in the dorsal motor nucleus of the vagus in the obex (Peters, Miller et al. 2000; Spraker, Balachandran et al. 2004; Keane, Barr et al. 2008), followed by centrifugal neural spread to peripheral sites. We were therefore surprised to find that PrP^{CWD} amplification was restricted to the obex of 4 out of 5 urine/feces inoculated deer. As with any test, there is the potential for poor sample quality to affect test results; this may explain the absence of detectable PrP^{CWD} in the lymphoid tissues of urine and feces inoculated deer. However, based on the abundance of PrP^{CWD} in lymphoid tissues of

saliva-inoculated deer, we are left with the interpretation that CWD prions may have bypassed amplification in the LRS of these deer. While these results are in contrast to the current concept of CWD pathogenesis (Sigurdson, Williams et al. 1999; Sigurdson, Spraker et al. 2001; Mathiason, Powers et al. 2006; Keane, Barr et al. 2008), absence of LRS involvement has been observed in natural cases of scrapie (Jeffrey, Martin et al. 2001; Jeffrey, Ryder et al. 2001; Jeffrey, Begara-McGorum et al. 2002; Siso, Jeffrey et al. 2009) and bovine spongiform encephalopathy (BSE) of cattle and sheep (Terry, Marsh et al. 2003; Wells 2003; Beekes and McBride 2007; van Keulen, Bossers et al. 2008). In scrapie-infected sheep, for example, the absence of scrapie amplification in the LRS as a result of host PrP genotype, limited dose exposure, tissue route/conduit, or prion strain selection are all supported by available evidence (Jeffrey, Martin et al. 2001; Jeffrey, Ryder et al. 2001; Jeffrey, Begara-McGorum et al. 2002; Siso, Jeffrey et al. 2009), and may also be plausible in cases of CWD. The deer included in our study were of two PrP genotypes at position 96, expressing either G/G or G/S, both of which have been shown to be LRS competent in the pathogenesis of CWD, although slower pathogenesis has been linked to 96S (Wolfe, Spraker et al. 2007; Keane, Barr et al. 2008). In naturally occurring cases of bovine spongiform encephalopathy, the BSE prion seems to exclusively utilize autonomic neural pathways for neuro-invasion, a defining characteristic of BSE (Terry, Marsh et al. 2003; Wells 2003; Beekes and McBride 2007; van Keulen, Bossers et al. 2008). It is possible that CWD strains may eventually be uncovered with BSE-like phenotypes which could be selected for by low dose exposure. Nevertheless, there have been no reports of PrP^{CWD} detection solely in the CNS of

infected deer. Thus the exact route by which CWD prions accessed the CNS of deer in the present study remains unidentified.

Detection of CWD prions in the present study samples relied heavily on sPMCA, with cervid transgenic mouse bioassay as a confirmatory assay. Given the risk of spurious positive results possible with multiple rounds of sPMCA, whether due to "spontaneous generation" or contamination (Deleault, Harris et al. 2007; Agrimi, Cosseddu et al. 2008; Barria, Mukherjee et al. 2009), we took great care to minimize this risk using approaches that parallel those to reduce cross-contamination in nested PCR. These precautions took two forms: avoiding cross-contamination during sample collection and initial processing, and prevention of intra-experimental cross-contamination. Animals were necropsied in order of approximate level of infection (based on inoculation material and known tonsil biopsy results), beginning with negative controls, followed by animals exposed yet biopsy negative throughout the study, and finally exposed, biopsy positive animals. Fresh necropsy instruments were used for each necropsy, with the central nervous system tissues removed at the completion of the necropsy. All study and control samples were then prepared in parallel using identical equipment and reagents. Normal brain homogenate substrates were prepared and loaded in sPMCA plates in a laboratory not used for prion study using disposable or sterilized equipment. Negative controls included multiple samples from both sham-inoculated cervids and uninfected Tg[CerPrP]-*tg5037* mice. In addition, the number of amplification rounds was limited to three to help ensure specificity. Perhaps most importantly, the positive sPMCA results identified in brain and lymph node homogenates

were confirmed by bioassay to provide support the conclusion that the sPMCA+ tissues did in fact harbor infectious prions.

In summary, we provide evidence for the presence of infectious prions in the brains of conventional prion-assay-negative deer orally exposed 19 months earlier to urine and feces from CWD-infected donor deer. This apparent low level of prion infection was amplified by sPMCA, confirmed by Tg[CerPrP] mouse bioassay, and detected only in the obex region of the brain. These results demonstrate the potential for CWD prion transmission via urine and/or feces, and highlight the application of more sensitive assays such as sPMCA in identification of CWD infection, pathogenesis, and prevalence.

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Chapter 3:

Detection of CWD Prions in Salivary and Urinary Tissues of Deer: Potential Mechanism for Prion Shedding

ABSTRACT

Saliva and urine are thought to play an important role in the transmission and pathogenesis of chronic wasting disease (CWD) in captive and free-ranging cervids. We have previously identified PrP^{CWD} in a variety of excreta using serial PMCA (sPMCA) and bioassay; however the source of infectious prions in urine and saliva has yet to be identified. In the present study, we applied sPMCA to tissues associated with saliva and urine production and excretion in an effort to seek proximal sources of prion shedding. Oropharyngeal and urogenital tissues, along with blood and obex from CWD-exposed cervids (comprising over 300 individual samples) were analyzed blindly in duplicate and scored based on apparent CWD burden. PrP^{CWD} was detected by 3 rounds of sPMCA in tissues associated with saliva and urine production and excretion, notably salivary gland and urinary bladder; whereas blood samples from the same animals and concurrent negative controls (n= 116 of 117) remained negative. Route of inoculation and CNS burden appeared to play an important role in terminal prion distribution, in that IV-inoculated animals and those with increasing CNS levels of PrP^{CWD} had higher and more widely distributed accumulation in excretory tissues. PMCA identification of PrP^{CWD} in oropharyngeal and urogenital tissues – in the absence of detection by conventional methods – may indicate the presence of protease-sensitive infectious prions in excretory tissues not revealed by assays employing PK digestion or other means to remove PrP^{C} reactivity. Thus evaluation of peripheral tissues via sPMCA may allow additional insights into prion transmission, trafficking, and pathogenesis.

BACKGROUND

Chronic wasting disease (CWD) is an efficiently transmitted prion disease of cervids (e.g. deer, elk, and moose), and is the only known prion disease affecting free-ranging, non-domestic animals. The origins of CWD are uncertain, but the disease has been present in wild cervid populations of northern Colorado and southern Wyoming for over 40 years (Williams and Young 1980; Williams and Young 1982). Since its discovery, CWD has been identified in both captive and free-ranging cervids in 17 states, 2 Canadian provinces, and Korea (Sigurdson 2008). With intensified national surveillance efforts, CWD has been detected in areas previously thought to be free of infection, including recent discoveries in Virginia and Missouri (www.promedmail.org 2010a; www.promedmail.org 2010b). The prevalence of CWD varies across North America, but can be as high as 30% in some areas of Colorado or in captive populations (Keane, Barr et al. 2008).

While much remains to be learned about the mechanisms of horizontal CWD transmission, a number of recent studies have shed some light on the potential routes of CWD shedding and transmission in nature. Initial experiments demonstrated conclusively that the environment and environmental fomites are capable of transmitting disease in both outdoor and later indoor cervid housing facilities (Miller, Williams et al. 2004; Mathiason, Hays et al. 2009). Subsequently, both saliva and blood were shown to efficiently transmit infection from CWD+ animals to naïve white-tailed deer (Mathiason, Powers et al. 2006). The role of urine and feces was less clear following these early experiments; however the use of transgenic mouse bioassay in the evaluation of excreta

from infected individuals, in addition to more sensitive evaluation of subclinically infected deer inoculated with urine and feces via PMCA, later demonstrated true infectious potential in both forms of excreta (Haley, Mathiason et al. 2009; Haley, Seelig et al. 2009).

The identification of infectious prions in bodily fluids represents an important step in understanding both the pathogenesis and transmission of CWD; however the proximal source of the agent in these excreta has not been examined in depth. A variety of organs and tissues are responsible for producing and excreting both saliva and urine. In cervids, saliva is an aqueous solution composed of a variety of proteins and electrolytes produced primarily by sublingual, submaxillary, and parotid salivary glands (Kay 1960; Fickel, Goritz et al. 1998). During the course of rumination, gastric fluids have also been shown to intermix with saliva produced in the upper gastrointestinal tract of ruminants (Beauchemin 1991). When evaluated cytologically, a number of different cell types may be found in this protein-laden fluid, including epithelial cells, lymphocytes, and neutrophils derived from the tongue, oral mucosa and associated lymphatic tissues (Kutta, May et al. 2006). Urine, on the other hand, is produced through active filtration of circulating blood by the kidneys, removing the majority of proteins in favor of excretion of metabolic waste, electrolytes, and small amounts of water (Maloiy and Scott 1969; Hove and Jacobsen 1975). Prior to being excreted, urine passes through the ureters to the urinary bladder and into the environment via the ureter; transitional epithelial cells and lymphocytes from each of these tissues are a common finding in cervid urine. Thus, there are many potential sites for the excretion of CWD prions into

both saliva and urine, and identification of all likely sources will assist our understanding of both pathogenesis and transmission in the natural host.

Although there has been anecdotal evidence of immunohistochemical (IHC) identification of prions directly in excretory organs and tissues, for example in the kidneys of scrapie-affected sheep (Siso, Jeffrey et al. 2008) and in ectopic lymphoid aggregates in CWD+ deer (Hamir, Kunkle et al. 2006), as well as lingual tissues of hamsters inoculated with the HY strain of transmissible mink encephalopathy (Mulcahy, Bartz et al. 2004; DeJoia, Moreaux et al. 2006), the ability to reliably identify protease-resistant forms of the prion protein in cervid excretory tissues using conventional IHC has proven difficult (Kitamoto, Mohri et al. 1989; Spraker, Miller et al. 1997; Foster, Parnham et al. 2001; Fox, Jewell et al. 2006; Balachandran, Harrington et al. 2010). Studies identifying genuine infectivity in excretory tissues is more limited, however, with reports of infectivity in parotid salivary gland of scrapie infected goats (Hadlow, Eklund et al. 1974) and kidneys of scrapie infected mice (Eklund, Kennedy et al. 1967). In these particular studies, which have yet to be replicated as broadly in other model systems, a variety of urinary and oropharyngeal tissues were unfortunately overlooked, leaving out crucial data on tissue infection during the course of prion pathogenesis.

Serial protein-misfolding cyclic amplification (sPMCA) is an assay that has proven utility in the detection of low levels of protease resistant, and potentially protease-sensitive, forms of the infectious prion protein, without the need for lengthy and expensive mouse bioassay experiments (Saborio, Permanne et al. 2001; Soto, Anderes et al. 2005; Kurt, Perrott et al. 2007; Haley, Mathiason et al. 2009; Haley, Seelig et al.

2009). In this study, we utilized a standardized sPMCA assay to detect PrP^{CWD} in various tissues associated with salivary and urinary excretion (e.g. salivary glands, tongue, kidney, ureter, and urinary bladder) from 28 experimentally challenged white-tailed deer, and compared relative tissue levels of individual animals with those found in obex.

MATERIALS and METHODS

Infected cervids:

Twenty-eight white-tailed deer (*Odocoileus virginianus*) were exposed to CWD from positive and negative sources in various forms (e.g. urine and feces, saliva, environmental fomites, blood, or brain tissue) and by various routes (e.g. orally, intravenously, intraperitoneally, intracranially, or through environmental exposure). Deer had been housed for previous transmission studies (Mathiason, Powers et al. 2006; Mathiason, Hays et al. 2009; Mathiason, Hayes-Klug et al. 2010; Mathiason, Hayes-Klug et al. (Under Review)), and all animals were inoculated and maintained in accord with Colorado State University IACUC guidelines. The sources of inoculum included terminally-ill mule deer (*Odocoileus hemionus*) of unknown PrP genotype (courtesy of Michael Miller, Colorado Division of Wildlife), or compatriot white-tailed deer utilized in previous and concurrent studies of either of two genotypes: homozygous for glycine (i.e. G/G) at cervid PrP position 96, or heterozygous at that position, with alleles for both glycine and serine (G/S). The deer were monitored for 16 – 27 months post inoculation (mpi), during which time the majority became tonsil-biopsy positive for CWD by immunohistochemistry (IHC). Duration of clinical disease prior to necropsy ranged from 0 (i.e. no clinical signs) to 72 weeks. At scheduled necropsy dates, or when exhibiting signs of terminal disease, deer were euthanized and subjected to an extensive necropsy using fresh instruments and collection vessels for each individual, at which time bodily fluids and an array of tissues were collected and frozen at -80°C. Animals that were not tonsil-biopsy positive for CWD prior to euthanasia were thoroughly evaluated for CWD infection after necropsy, which included IHC and western blotting (WB) of neural and

lymphoid tissues which have been shown to be early diagnostic sites for CWD (e.g. obex, retropharyngeal lymph nodes, and tonsils). A complete description of animals, inoculation routes and sources, CWD status, time of first tonsil biopsy positivity in months-post-inoculation (mpi), incubation period at necropsy (in mpi), and duration of clinical signs (in weeks) is shown in Table 3.1.

Table 3.1: Summary of source animals with tissues evaluated by sPMCA

Animal ID	Inoculum	Route of Exposure	PrP Genotype (position 96)	CWD Tonsil Biopsy Status	Tonsil Biopsy(+)†	Incubation period at Necropsy§	Duration of Clinical Signs¶
144	Saliva	PO	G/G	(+)	12	26	6
113	Saliva	PO	G/G	(+)	12	19	NA
132	Saliva	PO	G/G	(+)	19	19	NA
122	Saliva	PO	G/G	(+)	12	19	NA
147	Saliva	PO	G/G	(-)	NA	16	NA
107	Saliva	PO	G/G	(+)	19	19	NA
4119	Blood	IV	G/S	(+)	12	24	38
347	Blood	IV	G/G	(+)	6	27	44
114	Blood	IV	G/G	(+)	19	19	NA
108	Blood	IP	G/G	(+)	12	19	NA
110	Blood	IV	G/G	(+)	12	19	NA
133	Blood	IV	G/G	(+)	12	26	50
137	Blood	IV	G/S	(+)	19	19	NA
146	Blood	IV	G/G	(+)	12	29	72
141	Urine/Feces	PO	G/G	(-)	NA	19	NA
150	Urine/Feces	PO	G/G	(-)	NA	19	NA
111	Urine/Feces	PO	G/S	(-)	NA	19	NA
134	Urine/Feces	PO	G/G	(-)	NA	19	NA
124	Urine/Feces	PO	G/S	(-)	NA	19	NA
4461	Environment	Unknown	G/G	(+)	15	19	NA
4129	Environment	Unknown	G/S	(+)	19	19	NA
106	(+) Brain	IC	G/S	(+)	12	22	12
121	(+) Brain	IC	G/S	(+)	6	26	24
123	(-) Brain	IC/PO	G/G	(-)	neg	19	NA
103	(-) Brain	IC/PO	G/G	(-)	neg	19	NA
4488	(-) Brain	IC/PO	G/S	(-)	neg	19	NA
4516	(-) Brain	IC/PO	G/G	(-)	neg	19	NA

† First tonsil biopsy positive result, in months post inoculation (mpi)

§ Incubation period in months post inoculation

¶ Duration of clinical signs in weeks

Study samples and preparation:

Obex, parotid salivary gland, tongue, kidney, ureter, urinary bladder, and blood were collected at necropsy and frozen at -80°C. Tissue samples were thawed briefly and 10 – 50mg of each were trimmed individually and homogenized as a 1% solution (w/v) in PMCA buffer (1% triton-X 100 [v/v], 5mM EDTA, and 150mM NaCl in PBS adjusted to a pH of 7.2) using a FastPrep™ tissue homogenizer for 60s at power setting 6.5. Whole blood samples remained suspended in 5% EDTA until sPMCA evaluation. All tissues were prepared in individual microcentrifuge tubes and homogenized in parallel in the same machine concurrent with both positive and negative controls. Sample preparation tubes were coded to allow for blinded evaluation by serial PMCA.

Preparation of normal brain homogenate for sPMCA:

Normal brain homogenate (NBH), the substrate for prion conversion *in vitro*, was prepared from *tg5037* mice in a room that had not previously been used for prion research. Following euthanasia and perfusion with 5mM EDTA in phosphate-buffered saline (PBS), whole brain was collected from naïve *tg5037* mice and placed on ice. Brain homogenates were prepared as a 10% (w/v) solution in PMCA buffer with the addition of Complete Protease Inhibitors (Roche Pharmaceuticals, Indianapolis, IN) using a dounce homogenizer. Homogenates were then centrifuged for 1 minute at 2000rpm and the supernatant frozen in single-experiment aliquots at –80ºC in a "prion-free" room until use in sPMCA. Each preparation was composed of brain from 4-6 mice to minimize the influence of the expression variation of CerPrP or other co-factors (Browning, Mason et al. 2004; Angers, Seward et al. 2009).

Serial PMCA of tissues:

Tissue homogenates from CWD-exposed deer were blindly assayed, in duplicate, for PrP^{CWD} by sPMCA. All test samples were prepared in parallel with tissue-matched positive and negative controls as a 1% homogenate in PMCA buffer as described above and subsequently spiked into normal brain homogenate for amplification as described previously (Kurt, Perrott et al. 2007; Haley, Mathiason et al. 2009; Haley, Seelig et al. 2009; Kurt, Telling et al. 2009). Ten µl of test or control tissue homogenate were added to 50µl of NBH and assayed, in parallel and in adjacent wells of a 96-well plate (USA Scientific, Ocala, FL); along with normal brain homogenate prepared from unexposed *tg5037* mice as additional, unseeded negative controls. Plates were then sonicated using an ultrasonic processor (Misonix, Farmingdale, NY) and incubated at 37ºC. Sonication parameters were set at 40s bursts at power level 7.0, followed by 30 minutes of incubation. Ninety two cycles of sonication were performed over 48 hours, with a 10µl aliquot transferred to 50µl of fresh NBH for serial amplification. Following each round of amplification, samples were evaluated by western blotting, as described below, for the presence of PrP^{CWD}. Each sample was given a score based on the number of rounds that particular sample was positive (a maximum of three in a three-round experiment), and the scores for each duplicate run were totaled to arrive at a final score, with "0" being the lowest and "6" being the highest score a given sample could receive. In each experimental run, between 25 – 50% of samples evaluated were tissue-matched negative controls. Additionally, 10 – 20% of the samples in a run were unspiked, normal brain homogenate negative controls. Over the course of sPMCA experiments, multiple NBH

preparations were used, with each preparation consisting of between 4 - 6 source transgenic mouse brains to minimize individual differences in [CerPrP] expression levels.

Serial PMCA of blood:

EDTA-preserved whole blood samples from CWD-exposed deer were blindly analyzed in duplicate as described above for tissues, with some modifications. Using the protocol initially described by Tattum et al (Tattum, Jones et al. 2010), we spiked 1µl of whole blood into 100µl of normal brain homogenate. Sixty µl of this preparation was added to a 96-well plate and analyzed by sPMCA. Sonication parameters were set at 20s bursts at power level 7.5, followed by 30 minutes of incubation at 37°C. One hundred forty-four cycles of sonication were performed over 72 hours, with a 10µl aliquot transferred to 50µl of fresh NBH for serial amplification. Each sample was WB-evaluated as described below for PrP^{CWD} signal following each round of amplification.

Serial PMCA of obex dilution series and approximation of tissue scores:

Preliminary dilution series of obex tissue from seven animals, deer #110, 111, 113, 134, 137, 144, and 4119, collected and processed as described above, were made by diluting each of the initial 1% obex homogenates into 10% normal Tg[CerPrP] brain homogenate (NBH) at 1:10 dilutions ranging from 10^{0} to 10^{-14} (i.e. overall dilutions of 10^{-2} - 10^{-16}). Prior to diluting, the initial 1% obex homogenates were sonicated for 20s at power setting 7.0 in an ultrasonic processor. Ten µl of each dilution was added to 50µl NBH and analyzed for three rounds of PMCA as described for tissues above. Each dilution then received a score following western blotting; these scores were then used to

estimate the relative log obex dilution equivalent (LODE) scores of individual tissues within animals. Correlation coefficients between obex dilutions and sPMCA scores were determined using a commercially available statistical software package (Microsoft Xcel 2007).

Western blotting (WB):

Following each round of sPMCA amplification, an aliquot of each sample was subjected to western blotting for evaluation of PrP^{CWD} signal. Fifteen µl of sample homogenate were mixed with 7µl of sample buffer (0.1% [v/v] triton-X 100 and 4%(w/v) SDS in PBS) and digested with 3µl proteinase-K at 500µg/ml (final concentration: 60µg/ml) for 20' at 37ºC followed by 10' at 45ºC. Eight µl of 4X running buffer were then added to the sample, followed by denaturation for 5' at 95ºC. Fifteen µl of this preparation were run on a pre-cast 12% SDS-PAGE gel (Invitrogen) in a Bio-Rad electrophoresis apparatus for 1 hour at 160mV. Samples were then transferred to a PVDF membrane (Millipore) for 1 hour at 115mV in a Bio-Rad transfer apparatus. PVDF membranes were subsequently probed with a PrP-specific monoclonal antibody (BAR224-HRP) diluted 1:20,000 in 5% (w/v) powdered milk in 0.2% Tween-20 in tris-buffered saline (TBST) for 1 hour. Following washing with TBST, immunoreactivity was detected using an enhanced chemiluminescent detection system (ECL-plus, Amersham Biosciences) in a LAS 3000 imaging system. (Fuji Photo Film, Fuji Inc, Valhalla, NY)

Evaluation of intra-run variability in duplicate experiments:

As all samples were run in duplicate, often on two different sonicating machines, we attempted to compare the intra-run variability in experimental results. A commercially-available calculator for categorical Cohen's kappa value was used to define the agreement of results between the two different experiments (Faculty.Vassar.edu 2009). As scores progressed linearly in the sPMCA evaluation, a linear weighted approach was used to calculate Cohen's kappa. Significance of correlation was determined using the method described by Landis and Koch (Landis and Koch 1977).

RESULTS

In an effort to determine the proximal source of prions in saliva and urine, and to add crucial elements to our understanding of CWD pathogenesis, we blindly evaluated, in duplicate, a range of organs and tissues associated with saliva and urine production and excretion for PrP^{CWD} amplification using a standardized sPMCA assay. These tissues were then scored based on number of rounds demonstrating amplification; individual tissue scores were then grouped according to source of inoculum and correlated with intra-animal obex dilution scores.

Detection of PrP^{CWD} in peripheral tissues of exposed deer by serial PMCA:

Using a serial PMCA protocol shown to amplify PrP^{CWD} from both central and peripheral tissues in previous experiments, we blindly evaluated an array of tissues, from CWD-exposed and naïve deer, associated with salivary and urinary production and excretion, including salivary glands, tongue, kidney, ureter, and urinary bladder for evidence of PrP^{CWD} accumulation. In total, over 300 individual samples were evaluated, including 117 negative control samples. Animals were initially grouped based on inoculum source (e.g. saliva, blood, urine and feces); specific tissue scores in each of these groups were averaged to determine mean tissue score for each group. Next, animals were grouped according to obex score, and associated tissue scores were then averaged to determine the mean tissue score for each corresponding obex score. In exposed animals, PrP^{CWD} was amplified to varying degrees in all tissues evaluated (Table

3.2); with amplification patterns dependent on source of inoculation (Figure 3.1) and apparent obex burden (Table 3.3). Animals inoculated IV or IP with whole blood demonstrated the most widespread distribution of amplified PrP^{CWD}, while PO inoculation of either saliva or urine and feces revealed a more limited distribution. Interestingly, the amplification in some peripheral tissues, such as salivary gland and urinary bladder, rivaled or eclipsed amplification observed in the obex (e.g. salivary gland and urinary bladder from deer 144, urinary bladder from deer 4461, etc.). Among 117 negative control samples, a single false positive was identified in a kidney section from deer #103. This particular sample was evaluated in four separate experiments, and received a total score of "1" in a single sPMCA experiment; no amplification was observed in the three remaining experiments.

Animal ID	Inoculum	Route of Exposure	Obex	Salivary Gland	Tongue	Kidney	Ureter	Urinary Bladder	Blood
144	Saliva	PO	6	6	2	0	2	6	0
113	Saliva	PO	6	6	0	0	0	5	0
132	Saliva	PO	6	0	0	0	0	3	0
122	Saliva	PO	4	0	0	5	0	1	0
147	Saliva	PO	4	0	0	1	0	0	0
107	Saliva	PO	1	3	1	0	0	0	0
4119	Blood	IV	6	6	2	0	5	4	0
347	Blood	IV	1	2	0	2	0	0	0
114	Blood	IV	6	5	0	4	2	6	0
108	Blood	IP	3	0	0	0	0	0	0
110	Blood	IV	6	6	3	5	3	6	0
133	Blood	IV	6	6	6	5	4	1	0
137	Blood	IV	4	0	0	0	0	1	0
146	Blood	IV	6	NA	1	5	2	6	0
141	Urine/Feces	PO	1	0	0	0	0	0	0
150	Urine/Feces	PO	1	0	0	0	0	0	0
111	Urine/Feces	PO	2	0	0	0	0	1	0
134	Urine/Feces	PO	4	0	0	1	0	0	0
124	Urine/Feces	PO	0	0	0	1	0	0	0
4461	Environment	Unknown	3	0	4	3	4	6	0
4129	Environment	Unknown	3	0	0	0	0	0	0
106	(+) Brain	IC	6	5	0	0	0	4	0
121	(+) Brain	IC	6	6	0	0	1	6	0
123	(-) Brain	IC/PO	0	0	0	0	0	0	0
103	(-) Brain	IC/PO	0	0	0	1	0	0	0
4488	(-) Brain	IC/PO	0	0	0	0	0	0	0
4516	(-) Brain	IC/PO	0	0	0	0	0	0	0

Table 3.2: Summary of sPMCA results in blood and tissues associated with production and excretion of saliva and urine in CWD-exposed deer. Scores varied among routes of inoculation and inoculation sources. Occasionally, peripheral tissues rivaled or exceeded those of the obex (i.e. deer #144 salivary gland and urinary bladder, tongue and ureter of deer #4461)

NA: sample not available.

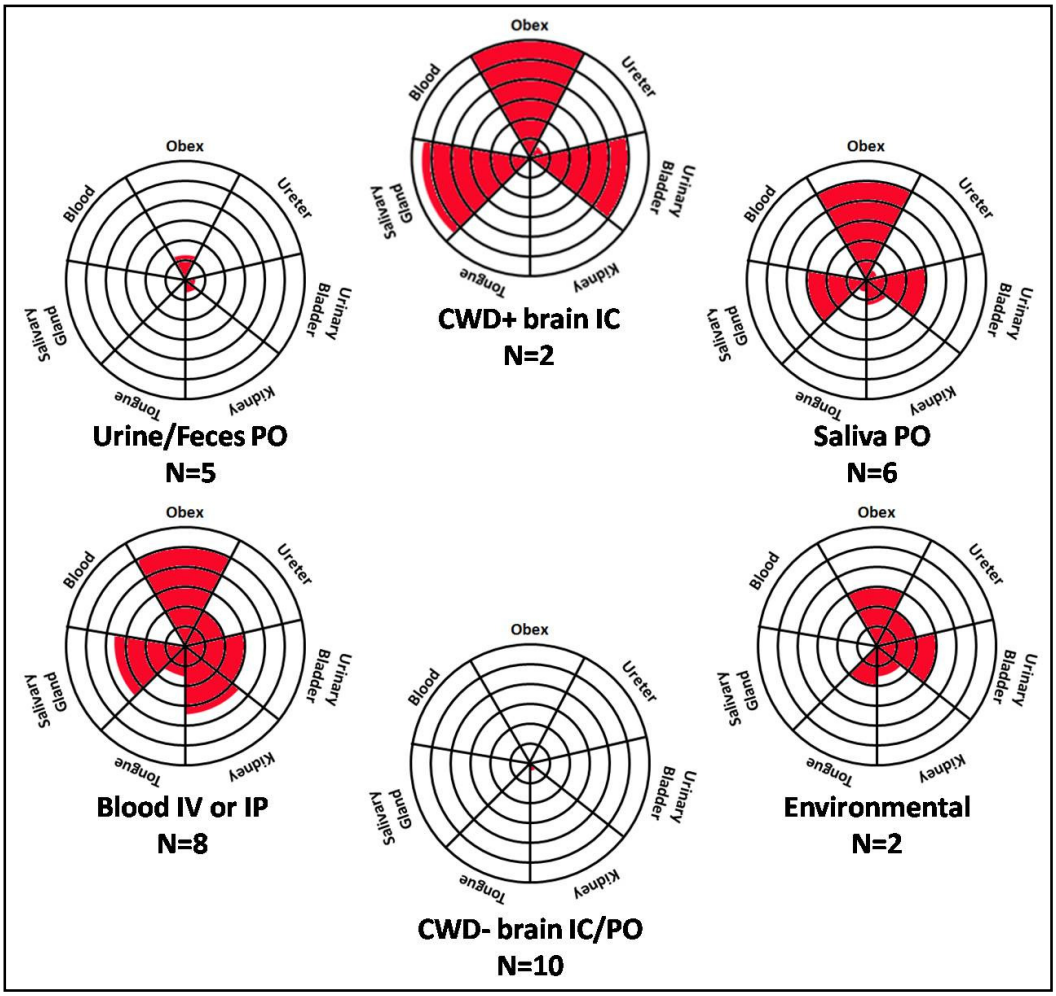

Figure 3.1: Radar plot summaries of tissue sPMCA results. Animals were grouped according to source of inoculum (blood, saliva, etc) and route of inoculation. Central concentric rings represent a score of "1," with scores progressing radially outward up to a score of "6." For each source of inoculum, colored wedges represent the mean individual score for that particular tissue. Patterns of distribution varied with both source and route of inoculum, with the most widely distributed patterns identified in animals inoculated with blood either IV or IP. Animals inoculated orally with either saliva or urine and feces had a much more limited tissue distribution, while tissues affected in animals inoculated IC with CWD+ brain were limited almost exclusively to salivary glands and urinary bladder.

Obex Score	1	2	3	4	5†	6
Salivary Gland	1.25	0	0	0	-	5.75
Tongue	0.25	0	1.3	0	-	1.6
Kidney	0.5	0	1	1.75	-	2.1
Ureter	0	0	1.33	0	-	2.1
Urinary Bladder	0	1	2	0.5	-	5.2
Blood	0	0	0	0	-	0

Table 3.3: Correlation of intra-animal obex scores with those of individual blood and tissue scores. Data represents average tissue scores within obex score categories. As obex score increases from "1" to "6," individual tissues show a corresponding trend of increase in most cases.

† In the course of sPMCA evaluation, no animals were identified with an obex score of "5."

Three-rounds of serial PMCA are not sufficient for PrP^{CWD} amplification in cervid whole blood:

Serial PMCA has been shown to be effective in the amplification of blood-borne PrP^{res} in various models of prion disease, including scrapie in both the natural host and mouse models. To date, however, no protocol has proven effective for amplifying PrP^{res} in the CWD model system – despite adequate evidence that blood and its components harbor infectious amounts of CWD prions (Mathiason, Powers et al. 2006; Mathiason, Hayes-Klug et al. 2010). Various sPMCA protocols have been described for blood evaluation (e.g. Castilla, Saa et al. 2005; Murayama, Yoshioka et al. 2007); however we chose a protocol that has recently been shown effective in a model of naturally-acquired

scrapie in sheep (Tattum, Jones et al. 2010). In an effort to demonstrate the feasibility of amplifying PrP^{CWD} in cervid whole blood, or alternatively to rule out blood contamination in tissue evaluations, we blindly examined twenty-eight whole blood samples from both CWD-exposed and naïve deer. In three rounds of sPMCA, all samples remained negative for PrP^{CWD} amplification (Table 3.2 and 3.3, Figure 3.1), ruling out blood contamination of tissues in a limited number of PMCA rounds, though perhaps not ruling out the feasibility of sPMCA to detect PrP^{CWD} in further rounds of PMCA.

Preliminary evaluation of obex dilutions to estimate tissue log obex dilution equivalent (LODE) scores:

Seven deer with variable levels of amplifiable PrP^{CWD} in both obex and peripheral tissues (deer #110, 111, 113, 134, 137, 144, and 4119) were selected for sPMCA evaluation of 10-fold obex dilutions. Dilutions of 1% obex homogenates ranging from $10^{0} – 10^{-14}$ (i.e. overall dilutions of $10^{-2} – 10^{-16}$) were evaluated in the standardized three-round PMCA assay, and each dilution was assigned a score as described for tissues above (Figure 3.2a [orally inoculated deer] and 3.2b [IV inoculated deer]). Individual peripheral tissues were then conservatively correlated with the lowest obex dilution receiving that particular score and assigned a log obex dilution equivalent (LODE) score. In the limited number of deer evaluated, tissue LODE scores were greatest in salivary gland and urinary bladder, which scored as high as 10^{-1} to 10^{-3}, respectively (Table 3.4). Trend lines were generated from the data by a mathematical software program (Microsoft

Xcel). Coefficients of correlation between these trend lines and the corresponding data ranged from 0.62 (deer #111) to 0.98 (deer #4119).

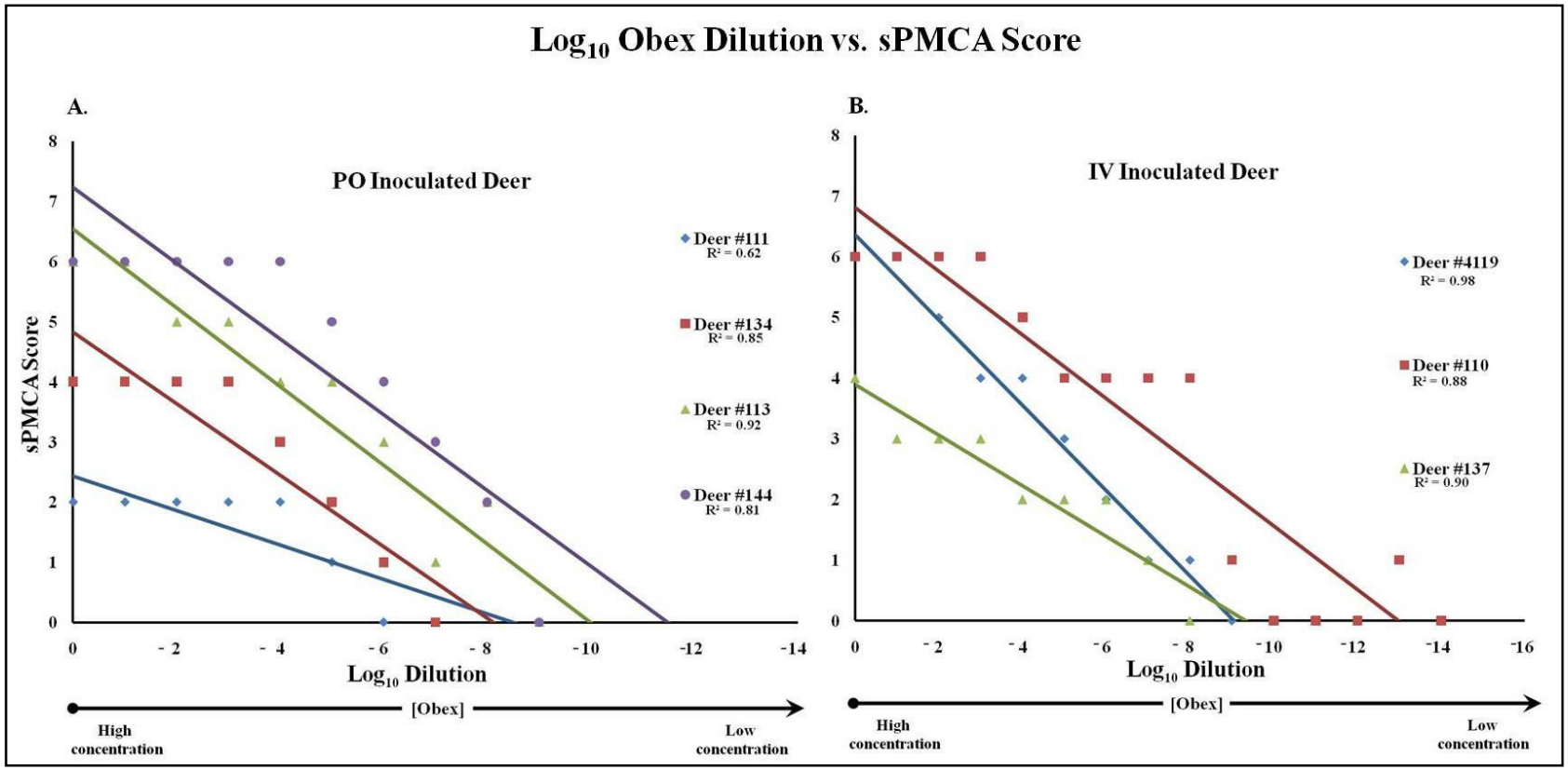

Figure 3.2: Comparison of 10-fold dilutions of obex to corresponding sPMCA scores in (A) orally inoculated deer and (B) IV inoculated deer. As dilution factor increased, sPMCA scores decreased. Coefficients of correlation between trend lines and corresponding data ranged from 0.62 (deer #111) to 0.98 (deer #4119).

Deer ID:	110	111	113	134	137	144	4119
Obex Score:	6	2	6	4	4	6	6
Tissue	Tissue LODE score						
Salivary Gland	10^{-4}	$<10^{-6}$	10^{-1}	$<10^{-7}$	$<10^{-8}$	10^{-3}	10^{-1}
Tongue	10^{-8}	$<10^{-6}$	$<10^{-9}$	$<10^{-7}$	$<10^{-8}$	10^{-8}	10^{-6}
Kidney	$<10^{-9}$	$<10^{-6}$	$<10^{-9}$	10^{-6}	$<10^{-8}$	$<10^{-10}$	$<10^{-9}$
Ureter	10^{-8}	$<10^{-6}$	$<10^{-9}$	$<10^{-7}$	$<10^{-8}$	10^{-4}	10^{-2}
Urinary Bladder	10^{-4}	10^{-5}	10^{-3}	$<10^{-7}$	10^{-7}	10^{-8}	10^{-4}

Table 3.4: Tissue LODE scores of individual oropharyngeal and urogenital tissues. Values conservatively represent the $\log_{10}$-fold dilution of obex required to achieve a score equivalent to that particular tissues score. Salivary gland and urinary bladder generally exhibited the greatest scores, approaching 10^{-1} to 10^{-4} obex dilution equivalents, respectively.

Individual intra-run sPMCA scores show a high level of agreement:

Using a commercially-available calculator for categorical Cohen's kappa, the sum of results available from blood and tissue sPMCA experiments were analyzed to determine the level of agreement between duplicate runs. A summary of the categories and scores achieved in duplicate runs on sonicators "A" and "B" are presented in Table 3.5. Because the progression of scores for each experiment is a linear one (i.e. from "0" – "3"), the kappa value was weighted linearly. The observed kappa value for the sum of sPMCA experiments was 0.80 (SE: 0.031; 95% CI: 0.74 – 0.87), which is considered as substantial to near-perfect agreement under guidelines proposed by Landis and Koch (Landis and Koch 1977).

Sonicator "A" \ Sonicator "B"	0	1	2	3
0	220	13	0	1
1	9	9	3	1
2	3	3	9	3
3	0	0	5	24

Table 3.5: Summary of scores achieved in duplicate sPMCA experiments. Tissues were evaluated in duplicate on two different sonicators – "A" and "B," receiving a score ranging from 0-3 in each replicate. Corresponding scores from duplicated experiments were tallied and evaluated categorically to derive a value for Cohen's kappa (0.80+/-0.031).

DICUSSION

Among the many described transmissible spongiform encephalopathies (TSE's) of animals and man, chronic wasting disease is unique in its relatively high levels of transmissibility. Using serial PMCA and transgenic mouse bioassay, we have previously demonstrated infectious prions in both urine and saliva, as well as in deer that remained conventional-test negative long after oral exposure to urine and feces (Haley, Mathiason et al. 2009; Haley, Seelig et al. 2009). While these studies demonstrate the important role of bodily fluids in horizontal spread of CWD, the source of infectious prions and the kinetics of shedding in excreta remain unknown.

In a number of historical, cross-sectional studies in CWD-endemic cervid herds, conventional immunohistochemistry and western blotting failed to demonstrate protease-resistant prions in any of the organs of production and excretion of either saliva or urine (Spraker, Miller et al. 1997; Fox, Jewell et al. 2006; Balachandran, Harrington et al. 2010). Only recently has the potential for a protease-sensitive, yet still infectious, form of the prion protein – denoted $sPrP^{Sc}$ – been identified (Colby, Wain et al.; Safar, Wille et al. 1998; Safar, Geschwind et al. 2005; Thackray, Hopkins et al. 2007; Jansen, Head et al. 2010); because of the necessity for harsh proteolytic or formic acid treatments, conventional assays used to screen peripheral tissues may be handicapped in their ability to detect this form of PrP. Anecdotally, however, protease-resistant prions have been identified in ectopic lymphoid aggregates in the kidneys of CWD-exposed deer, but no conclusions could be drawn the infectivity of this particular sample and on the likelihood this phenomenon may play a role in prionuria in nature (Hamir, Kunkle et al. 2006).

Previous studies in hamsters have also linked nephritis with concurrent prionuria (Kariv-Inbal, Ben-Hur et al. 2006), a condition demonstrated in those deer whose urine was pooled in my initial bioassay studies (Haley, Seelig et al. 2009). Using IHC, protease-resistant prion has also been demonstrated in a variety of lingual tissues, including lingual epithelium, in hamsters inoculated with the hyper (HY) strain of transmissible mink encephalopathy (DeJoia, Moreaux et al. 2006), while early mouse bioassay experiments have also identified infectious prions in the salivary glands of scrapie-exposed goats (Hadlow, Eklund et al. 1974). Each of these studies, whether examining oral or urogenital tissues, again raises questions regarding the ultimate mechanisms involved in prionsialia and prionuria – specifically: (1) from where do infectious prions arise in these bodily fluids; (2) are infectious prions transmitted in a cell-free or cell-associated form; and (3) do infectious prions transmitted in excreta occur in their traditional, protease-resistant form, or a more elusive protease-sensitive species?

Based on our previous studies incorporating sPMCA to amplify very low levels of PrP^{CWD} in nervous and lymphoid tissues of exposed deer (Haley, Mathiason et al. 2009), we attempted to further evaluate a number of organs and tissues associated with saliva and urine production and excretion, including salivary gland, tongue, kidney, ureter, and urinary bladder. As sPMCA has also been shown capable of amplifying both protease-resistant and -sensitive forms of PrP^{Sc} (Pastrana, Sajnani et al. 2006), the absence of protease-resistant forms in these tissues would not preclude positive amplification. After blind evaluation by sPMCA, in duplicate, we were able to identify PrP^{CWD} in each of these tissues to varying degrees, with experimental results showing high levels of agreement between runs. Tissue variation was related to both the source and route of

inoculate an animal was exposed to (e.g. IV whole blood, PO urine and feces, etc), as well as the apparent PrP^{CWD} burden within the obex of the individual animal. Tissue distribution variation with route of inoculation has been described in both viral and bacterial infections (Singh, Singh et al. 1987; Bravata, Holty et al. 2007; Prentice and Rahalison 2007; Maisner, Neufeld et al. 2009), so this finding is perhaps not surprising. Likewise, in the case of neurotropic viruses, peripheral tissue levels of virus often positively correlate with central nervous system burden (Charlton, Casey et al. 1983). A detailed evaluation of the tissue distribution of PrP^{CWD} in naturally-occurring cases may reveal patterns corresponding with those described in this study, which may help identify prevailing routes of transmission occurring in nature. Additionally, these findings point to an increased risk of bodily fluid transmission with the progression of clinical disease.

What the findings of this study do not reveal is the sequential appearance of amplifiable prions in peripheral tissues. It has often been argued that TSE's follow a pattern of centripetal spread from the periphery to the central nervous system (via either the peripheral nervous system or the lymphoreticular systems), followed by centrifugal spread back to peripheral organs (Glatzel and Aguzzi 2000; Dormont 2002; Bartz, Dejoia et al. 2005; Unterberger, Voigtlander et al. 2005; Beekes and McBride 2007; van Keulen, Bossers et al. 2008). The results of this study argue that peripheral infection may occur concurrently with central invasion – given that peripheral levels often rival or exceed those of the obex, long considered a "gold standard" tissue for the definitive identification prion infection of deer and other ruminants (Wells, Hancock et al. 1989; Ersdal, Ulvund et al. 2003; Joly, Samuel et al. 2006; Keane, Barr et al. 2008). The amplification ability of PrP^{CWD}, either *in vivo* or *in vitro*, may explain some of the scoring

variation between tissues, though compared to obex, the relative distributions would be expected to be more consistent. We therefore evaluated log obex dilution equivalent (LODE) scoring of peripheral tissues, which conservatively demonstrated that, in most cases, excretory tissues, notably salivary gland and urinary bladder, score several log-fold dilutions lower than those observed in undiluted obex. To further understand the centripetal and centrifugal dissemination of CWD prions, the present findings warrant a sequential evaluation of the peripheral prion distribution of via sPMCA – both within excretory tissues as well as nervous and lymphoreticular conduits – of serially-exposed animals.

In summary, this study demonstrates for the first time amplifiable PrP^{CWD} in various organs and tissues associated with prionsialia and prionuria. The ultimate source and mechanism of release into bodily fluids remain unknown, though elevated levels in both salivary gland and urinary bladder provides strong evidence that these tissues play a crucial role in prion excretion. In addition, the source and route of inoculation weighed heavily on the terminal peripheral distribution of PrP^{CWD}, as did an individual's apparent central nervous system burden. Finally, while this discovery provides evidence for prion invasion of peripheral excretory tissues, the timing of infiltration during CWD infection and the protease resistance profile of these prions warrant future studies in serial pathogenesis and detection of alternate infectious prion species.

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CONCLUSIONS AND FURTHER DIRECTIONS

Through the investigation of terminal bodily fluid samples and tissues of CWD-exposed white-tailed deer, I have shown that: (1) both saliva and urine harbor infectious CWD prions, as demonstrated in Tg[CerPrP] bioassay and serial PMCA, (2) serial PMCA is sufficiently sensitive in detecting PrP^{CWD} in conventional test-negative tissues, and these tissues also harbor true infectivity through the transfer of infection in the Tg[CerPrP] bioassay system, and (3) serial PMCA is capable of detecting PrP^{CWD} in tissues involved in the production and excretion of saliva and urine, notably salivary gland and urinary bladder, and the levels of PrP^{CWD} in these organs correlate both with source and route of inoculum as well as central nervous system burden.

Although I have obtained preliminary data on the relative peripheral tissue levels of PrP^{CWD}, a full evaluation of the obex of deer used in this particular study is necessary. I also plan to evaluate other tissues potentially involved in fecal shedding by sPMCA, including elements of the gastrointestinal tract. The information gained through continued investigations of these tissues will provide further information on the pathogenesis of CWD and the dynamics of peripheral infection. Even at our present level of understanding, though, the data raises significant questions surrounding the role of peripheral tissues in either centrifugal or centripetal dissemination of CWD prions to or from the central nervous system, which mandate study of the kinetics of peripheral infection in naturally exposed deer. Serial necropsies of infected deer, followed by a thorough evaluation of peripheral tissues via sPMCA (including organs involved in production and excretion of urine, feces, and saliva, as well as neural and lymphoid

tissues) would allow a "moving picture" analysis of the pathogenesis of CWD, as opposed to the static images of pathogenesis we now have for prion diseases.

While I feel confident that I have generated significant information on the relative levels of PrP^{CWD} in both central and peripheral tissues using both a crude, semi-quantitative sPMCA scoring system and an approach utilizing log-obex dilution equivalent scores, there is very little information on the relationship between the apparent amplification ability of a given tissue and its actual infectivity, i.e. how does the amplification level of a tissue correlate with its infectivity? In the second chapter, I evaluated tissues both by sPMCA and Tg[CerPrP] bioassay; to my knowledge this is the only manuscript that at least superficially addresses a correlation between amplification and infectivity in either unknown or conventional test-negative samples. To further evaluate the relationship between sPMCA amplification and infectivity, a large-scale evaluation of CWD in endemic areas is warranted. To that end, I have begun blindly evaluating a small subset of Rocky Mountain elk (*Cervus elaphus*) from Rocky Mountain National Park, where the prevalence of CWD approaches 11% in this species (Jenny Powers, personal communication). Archived tissue samples from this subset of animals include obex, recto-anal mucosa associated lymphatic tissue (RAMALT), tonsil, and retropharyngeal lymph nodes, and each of these has previously been evaluated for PrP^{CWD} by IHC. By evaluating individual tissues blindly by sPMCA and determining their respective scores, a data base of sPMCA and IHC results from a naturally exposed population may be obtained. This information can then be used in the design of transgenic mouse bioassay experiments, from which incubation periods can then be correlated to both IHC and sPMCA results. Bioassay, the gold standard for prion

presence in a particular sample, would also prove useful in the direct comparison of the sensitivity and specificity of both IHC and sPMCA, and could additionally assist in defining the true prevalence of CWD in the Rocky Mountain National Park elk herd.

While the identification of CWD prions in peripheral tissues is an exciting discovery that may lead to future understanding of CWD and prion disease pathogenesis and transmission, the "Holy Grail" of prion amplification assays is to be the first to apply it in the early identification of TSE infection in clinically relevant samples, specifically urine, saliva, or blood samples. In the first chapter, I demonstrated sPMCA amplification of PrP^{CWD} in a concentrated urine sample from a pooled set of animals; this report is still a long way from unearthing that Holy Grail. The repeated, blinded amplification of PrP^{CWD} in biological fluids has proven elusive over the course of the research presented in this dissertation, despite the apparent ease of amplification in tissues. Perhaps this is related to either the conformation or cellular environment present in tissues. I believe successful prion amplification in bodily fluids will first require development of the sPMCA assay for use in either cell culture or yeast models, where the sPMCA milieu may be specifically altered and optimized for cellular suspensions. Until then, I am fortunate to have a wealth of bodily fluid samples from the National Park Service, in addition to those tissue samples described above, with which to continue optimization of sPMCA in excreta.

Based on the studies presented in this thesis, it is my firm belief that sPMCA has a sensitivity far exceeding that of conventional IHC and western blotting. It seems plausible that the enhanced sensitivity is related to the apparent ability of sPMCA to amplify a protease-sensitive form of PrP^{res}, denoted "$sPrP^{res}$." This isoform of the

infectious prion protein was first identified through the use of the conformation-dependent immunoassay (CDI), which takes advantage of variable epitope exposure in the infectious prion protein and avoids the need for protease digestion. Because traditional, *in vitro* prion detection methods rely on harsh proteolytic treatment steps to discriminate PrP^{res} from the normal cellular isoform, it is likely that $sPrP^{res}$, if truly present *in vivo*, goes unnoticed in subclinical animals, their peripheral tissues and bodily fluids. What is certain is that a complete understanding of the role of $sPrP^{res}$ in pathogenesis and transmission is necessary if we ever hope to control the unchecked spread of naturally occurring prion diseases.

Finally, the research presented in this thesis, and the future avenues I hope to explore, will provide us with crucial information on the pathogenesis of CWD that, like scrapie in sheep, has the incredible ability to spread efficiently between susceptible members of the host species. As mentioned previously, though, CWD and scrapie seem to be unique in this aspect among the prion diseases, while prion diseases factoring more prominently in the public eye, like Creutzfeldt-Jakob disease (CJD) and bovine spongiform encephalopathy (BSE), seem to only rarely transmit horizontally – usually only as a result of human intervention (e.g. blood transfusions or the feeding of ruminant meat and bone meal). Parallel studies of the pathogenesis and peripheral dissemination of natural cases of CJD and BSE are warranted to uncover why it is that these agents lack efficient mechanisms of transmission, shedding further light on how CWD and scrapie accomplish this feat.

The studies comprising this thesis have contributed to furthering the understanding of the transmission and pathogenesis of chronic wasting disease.

Moreover, these studies lay an important foundation for future CWD studies, which will answer questions that enhance our understanding of the various forms of the prion protein in all species affected.