DISSERTATION

CHRONIC WASTING DISEASE STRAIN DIVERSITY, DISTRIBUTION AND TRANSMISSION

Submitted by

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ABSTRACT

CHRONIC WASTING DISEASE STRAIN DIVERSITY, DISTRIBUTION AND TRANSMISSION

Chronic wasting disease (CWD) is an invariably fatal prion disease affecting captive and free-ranging cervids, including white-tailed deer, mule deer, moose, elk and reindeer. Since the initial discovery of the disease in the 1960's, CWD has spread across the US and Canada, South Korea, and, most recently, Europe. While some outbreaks of CWD were caused by transport of infected animals from endemic regions, the origin of CWD in other epizootics is unclear and not all outbreaks have been characterized. Previous studies have shown that there are multiple strains of CWD; however, the continuous spread and the unclear origin of several outbreaks warrant continued surveillance and further characterization of strain diversity. Moreover, studies implicating extraneural prions as more zoonotic motivated us to examine within-host prion strain diversity. The overarching goal of the work presented here was threefold: 1) address CWD strain differences between lymphoid and brain tissue from the same animal, 2) assess if there are any differences in CWD from either within or between contiguous and non-contiguous outbreaks and 3) address aspects of plant-vectored CWD transmission. The work presented here has important implications for understanding strain diversity within and between deer, as well as identifies samples that appear to be novel strains that warrant follow up assessment. Finally, we show how plants may be playing a role in vectoring infectious prions shortly after exposure. This research has important implications for our understanding of prion strain diversity and distribution as well as adds insight to plant-vectored prion transmission.

First, we assessed differences between lymph node-derived and brain-derived prions from within the same animal to characterize strain differences within a single animal. To do this, we assessed isolates using biochemical techniques including electrophoretic mobility, glycoform ratio and conformational stability. Interestingly, we found that there were biochemical differences between lymph node and brain isolates, novel intermediate conformations of the prions in the brain (but not the lymph node) and increased variability in the lymph node-derived prions. Collectively, these results suggest that there are more diverse prion strains in the periphery and are distinct from neurological prions. The

research discussed here advances our understanding of the differences between lymph node-derived and brain-derived prions.

In addition to within-host strain comparisons, we also wanted to assess biochemical strain differences from naturally infected cervid species. Numerous studies have examined CWD strains upon passage into transgenic mouse models. For the purposes of our research, we wanted to examine CWD strains from the natural host for a number of reasons: 1) bioassay is expensive and time consuming, making strain characterization challenging, 2) research indicating that host factors other than PrP^C may be influencing strain characteristics and 3) to determine if we could detect dramatic biochemical differences in strains, thereby providing an easier method to determine CWD strain prevalence in cervid populations without bioassay. Because the origin of CWD is unknown and some outbreaks of CWD have no clear exposure/connection to ongoing CWD outbreaks, this research would provide insight into the evolution and origin of CWD. Here, we show that there are some cases of CWD that present with novel biochemical characteristics that distinguish them from other CWD isolates. These instances suggest a new strain has emerged or that there is differential evolution in these subpopulations. Importantly, this work highlights that there is a lot more variability CWD biochemical characteristics than previously described.

As a part of the strain typing project, two samples were received from captive white-tailed deer in Texas. These samples immediately proved to be a challenge to work with because they were behaving in an unusual way in our biochemical strain typing assays. In short, these isolates behaved in strange ways depending on the detergent class with which they were being digested. Because there was no known introduction of CWD to this captive herd, we were suspicious that we were seeing a novel strain of CWD. Isolates were passaged into cervid and human PrP mice. Upon passage, these isolates looked like classical CWD in Tg33 mice and, fortunately, don't appear to have any zoonotic transmission potential into human PrP mice. Importantly, this work highlights that CWD can present in a unique way in a cervid host but cause a classical-type disease in transgenic animals.

Finally, we examined the role of plants to transmit CWD. Previous research implicated plants as having a possible role as a vector in prion transmission. We built upon this previous research by using CWD prions rather than hamster prions and a different plant model. The research presented here will

show that plants are able to uptake prions shortly after exposure, but that these prions are no longer detected by 72 h. The work presented here implicates plants as potential CWD vectors in the short term.

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DEDICATION

This work is dedicated to my incredible daughter, Elise. May you always find happiness wherever you go.

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Chapter 1:

Introduction

Stumbling to the finish line: discovery of the prion

Prions are the causative agent of group of diseases known as transmissible spongiform encephalopathies (TSEs). While the nature of the infectious agent of scrapie was elegantly revealed by Nobel Laureate Stanley Prusiner a mere 39 years ago, prion diseases had been observed hundreds of years before the discovery of the infectious nature of the disease[1]. Notably, scrapie was observed in sheep as early as 1732 and was first recognized in goats in 1942[2–9]. While scrapie was recognized across Europe and went by many names, scrapie ultimately stuck as the name of this curious affliction that caused sheep to rub themselves against fences and rub their wool off. Ultimately, all the scraping sheep would die from a progressive neurological disease. While the cause of scrapie wouldn't be revealed for many years, Cüille and Chelle proposed that scrapie was perhaps caused by a slow virus in 1936 [5–7].

In 1944, evidence had emerged that the infectious component of scrapie was unlike anything that had been observed before when a vaccine against louping-ill virus ended up causing scrapie in recipient sheep. To generate the vaccine, sheep were inoculated with the louping-ill virus and brains were later collected from these animals and added to 35% formalin solution to prepare the vaccine, which was administered subcutaneously to sheep. A specific batch (batch "number 2") of the vaccine prep was found to be associated with an outbreak of scrapie in vaccinated flocks, providing some of the first evidence of the transmissibility of an infectious agent causing scrapie. Because of how the vaccine was prepared it was also apparent that the infectious agent was extraordinarily resistant to formalin[2,10]. On average, 10% of the vaccinated flocks were infected with scrapie, with incidence ranging between 1-26%[10].

The nature of scrapie was confusing for many studying the disease at the time. There were features of the disease that appeared to be hereditary[3]. Indeed, different breeds of sheep appeared to have differential susceptibility and selective breeding protocols for resistant sheep was somewhat successful in controlling scrapie, both powerful points to support the hereditary hypothesis for the nature of the disease.

Contrary to the hereditary basis for the disease, there was also evidence of “pasture transmission” where small groups of sheep affected with scrapie were housed in a paddock and then a group of 26 healthy ewes were allowed to graze. Affected and unaffected animals were allowed to alternatively graze for periods of 3-4 days and care was taken to never comingle the two groups of sheep. After a period of 3.4 years, some of the ewes become infected with scrapie, providing evidence of “pasture transmission” of the agent[11]. In light of transmission in the pasture and the vaccine transmission, it was proposed that scrapie was likely a filterable virus affecting the CNS with a prolonged incubation period first in 1938[5] and the hypothesis was again amplified in 1950s[3,12].

While hereditary and infectious origins of scrapie were being investigated, reports of Kuru, a novel, neurodegenerative disease, was reported in the Fore people of Papua New Guinea by Gajdusek and Zigas in 1957[13,14]. A brilliant observation by Hadlow in 1959 noted the similarities between the disease presentation and pathology between scrapie and kuru and suggested that the two diseases may be related[15,16]. This observation prompted experiments in chimpanzees to assess the transmissibility of the kuru agent. Chimpanzees were intracerebrally inoculated with brain material of individuals affected with kuru. All the infected animals succumbed to disease with a presentation and pathological findings remarkably similar to that of Kuru in humans[17]. Two years later, Creutzfeldt-Jakob disease was successfully transmitted to chimpanzees as well[18].

The findings that suggested scrapie and Kuru were both transmissible diseases prompted an explosion of research to characterize the infectious agent. Many of the earliest studies focused on scrapie, the archetype of prion disease. Scrapie in goats was found to present with two distinct forms of the disease, one presentation that was characterized by a scratching and “nibbling” syndrome and another disease presentation that was drowsy and uncoordinated[19–21]. A major step forward in scrapie research was achieved by Chandler in 1961 when he was able to successfully transmit the scrapie agent from a “drowsy” goat to swiss mice, causing a pathological finding of spongiosis and reactive astrogliosis, similar to the pathological findings from scrapie sheep[21,22]. Additional passages into mice also proved to be successful[23] and would prove to be extraordinarily valuable for the characterization of the scrapie agent. Once the mouse model was developed, research began in earnest to understand this enigmatic scrapie agent.

Numerous inactivation studies were conducted to elucidate the nature of the scrapie agent. Scrapie agent from infected mice were found to be resistant to heating (but a loss of infectivity seen when heated for long periods at 100°C), with no impact of freezing on infectivity[24]. Attempts to inactivate the scrapie agent with UV and ionizing radiation[25,26] were unsuccessful and predicting the size of the agent with ionizing radiation indicated that the agent was of extraordinarily small size[25,27]. Additional experiments found that heating, lipase, DNase, RNase, detergents and salts were all unable to abrogate scrapie activity [24–26,28,29]. Further work with formalin verified the findings from the accidental vaccine scrapie transmission and the agent was found to be transmissible after formalin treatment[30]. Additional stability studies would show that the scrapie agent was susceptible to periodate, urea, phenol, arcton 113, trypsin and papin[28]. These experiments, in addition to the vaccine fiasco, were some of the first pieces of evidence that the agent may not be viral in nature, or if indeed viral, unlike anything that had been previously noted. The lack of the scrapie agent to cause an immune response or have acute pathology set the agent apart from typical neurological viruses and perhaps should have also been a hint that this agent was of a unique infectious nature[31].

The first murmurs of an infectious protein

The collective research had thus far demonstrated that spongiform encephalopathies like scrapie (and kuru) did have aspects of an infectious agent, but if the infectious component of the disease was viral in nature as many suspected, it was unlike anything that had been characterized to date. Scientists began to postulate that the scrapie agent could replicate without any associated nucleic acid[26,32,33]. The central dogma of molecular biology had only recently been "cracked" a mere 15 years prior with X-ray crystallography of Rosalind Franklin and suggesting a process by which a protein was replicating and causing disease with a nucleic acid component was practically heretical [34]. The conclusions of Alper and Cramp eloquently discuss the surprising findings of inactivation experiments and expand on their work to imply that the scrapie agent doesn't rely on nucleic acid to replicate and may be reliant on association with a protein[26]. Gibbons and Hunter provide an exquisite summary of the thoughts in the field about the nature of the scrapie agent, making an argument for protein and other possibilities. They do, however, acknowledge the contradiction with modern molecular biology and question how a protein-encoded disease could fit in with the current understanding of molecular biology[32]. They also point out the

surprisingly “sterile” infection of the scrapie agent and question how a protein with such resistance to ionizing radiation could *not* be antigenic. It is the hypotheses of Griffith, however, that are most striking because of how he so eloquently incorporates a protein-basis of the scrapie agent with the central dogma of molecular biology. Incredibly, his hypothesis of two forms of a protein synthesized by the host, upon which a nucleation reaction can occur, is remarkably close to what we understand today of the nature of prion disease, replication and epigenetic transmission[33]. In one of the many experiments undertaken to understand the infectious agent, Hunter *et al.* would postulate that if the nature of scrapie was protein in origin, something much more complicated would need to be going on. They also, much ahead of their time, note that “…the scrapie agent may exist as an integrated membrane structure differing only in detail from similar structures present in the normal cell”[28]. Later, Lewin would again propose that scrapie and other slow viruses could possibly provide insight into a peptide (or other biological macromolecule) that wouldn’t necessarily break the central dogma of molecular biology but would add to the complexity of what was understood about life[35]. Today, we know that these hypotheses were most closely related to the truth, but the studies to address these hypotheses were challenging and nothing had proven unequivocal enough to cause wide-spread support for an infectious protein.

Not quite ready to break the central dogma

Early work on the genetic control of scrapie in mice found that there was genetic control for scrapie. The gene was named *sinc* for scrapie incubation time and two alleles were noted that made animals either susceptible or resistant to the onset of scrapie[36,37] . There was still extraneural accumulation of prions and the only difference was the time it took for the prions to reach the brain[36,38], similar to the nature of chronic wasting disease susceptibility and resistance seen today[39].

While the proteinaceous nature of the scrapie agent was being somewhat entertained in the field, another popular hypothesis at the time was the membrane hypothesis, which was formed in response to the unusual properties of the prion strain. Early work had found scrapie infectivity associated with plasma and mitochondrial fractions from scrapie-infected brains[40]. The membrane hypothesis suggested that the infectious nature of scrapie was associated with a combination of macromolecules as a part of the membrane. Research that found infectivity associated with the plasma membrane and mitochondrial fractions were supposedly evidence for this, as well as data indicating that membrane disrupting actions

of SDS could be proof of a membrane-infective agent[29,41–44]. The discovery of viroids in 1972 led to some speculation of scrapie being caused by a small viroid[45], but this was contradicted by evidence that RNases had no effect on infectivity[46]. Despite the copious evidence that the infectious nature of scrapie wasn't a virus, there were still some hold outs that it was a novel, yet undescribed slow virus or an infectious membrane.

The prion is born

Finally, this new kid named Stanley showed up and synthesized much of the research that had been done and revealed the protein-only nature of the scrapie agent through a series of excellent experiments. First, he found that the sedimentation properties of the scrapie agent from infected mice were unique and different from uninfected mice and that the protein was insoluble and was independent of membranes, casting doubt on the membrane hypothesis[47]. Then, because multiple conformers of the protein associated with infectivity were found, it was postulated that there was a hydrophobic protein associated with scrapie infectivity. The hydrophobicity of this protein was proposed to account for the lack of antigenicity and suggested aggregation potential of the scrapie agent. The infectious nature of the fraction that was isolated with ribosomes led them to suggest that a protein is a critical aspect of scrapie infectivity[48,49]. Finally, Pruisner demonstrated, unequivocally, the proteinaceous, infectious nature of the scrapie agent, called a prion[1]. Prusiner continued his exceptional work characterizing prions and found microheterogeneity in the scrapie prions, noted proteinase K (PK) resistance and imaged rod-like structures from scrapie-infected brains by EM[50]. He would later show that PK-resistance was only associated with infectivity[51] and that scrapie prions aggregate into rod like structures that stain like amyloid, and suggested that the plaques seen in TSEs are composed of prions[52].

To identify if there was any nucleic acid associated with scrapie, studies looking at mRNA or DNA of the scrapie prion found that mRNA was present in both infected and uninfected brains, implicating the prion protein as a normal constituent of mouse and hamster brain and wasn't found in purified prion fractions, indicating that the nuclei acid was not carried around by the infectious agent[53–55]. Together, this work suggested the presence of this protein in a normal situation but a change of conformation in a diseased state.

In 1990, Pruisner *et al.* proposed a model where the misfolded, prion protein was serving as a template upon which it would convert PrP^{C} into a misfolded form[56]. In 1991, Caughey used infrared spectroscopy to determine the PK resistant core of PrP^{Sc} was made largely of beta sheets[57]. In 1993, excellent work was done to demonstrate the three-dimensional changes that occur in the prion protein. Pan demonstrated the conversion of the prion protein, made up primarily of alpha helices to beta sheets and demonstrated the conformational transition that was requisite for prion transmission and Safar correlated the beta sheet structure with infectivity[58,59]. This conformational change from alpha-helical to beta-sheet is accompanied by protease resistance, insolubility, and increased resistance to denaturation.

With all the mounting evidence, there were still some holdouts on the protein-only hypothesis, but additional work to demonstrate the requisite nature of PrP^{C} was mounting. Mice that didn't express any level of endogenous PrP^{C} were found to be entirely refractory to prion infection[60,61], demonstrating the requisite nature of PrP^{C} and nearly solidifying the protein-only hypothesis. Spontaneous generation of prions both *in vitro*[62] and *in vivo*[63], followed by transmission of prions from recombinant bacterially produced prions[64] solidified the prion-only hypothesis, which is (nearly) ubiquitously accepted today.

Zombies are here: Human and animal TSEs

Scrapie

Scrapie is a prion disease affecting sheep and goats, as discussed above. While there are still cases today, scrapie eradication programs and selective breeding has created a situation in the US where scrapie is mostly controlled[65]. There are multiple scrapie genotypes that control susceptibility and resistance to disease, with ARQ being most susceptible and VRR most resistant to classical scrapie. While most resistant to classical scrapie, VRR animals are more susceptible to atypical scrapie[66]. With the exception of Australia and New Zealand, classical scrapie has a worldwide distribution. Scrapie is quite transmissible and there is evidence of scrapie being transmitted to susceptible lambs *in utero*, in milk and birthing materials[66–69]. The prolonged survival in pastures can also result in horizontal transmission[11,70].

Kuru

As discussed above, the brilliant observation of Hadlow[15] linking Kuru and scrapie pathology to begin the grouping of TSEs was paramount to our understanding of prion diseases. Kuru was a disease

affecting the Fore people of Papua New Guinea[14]. It was later found that ritualistic cannibalism was the culprit behind the disease. Because women and children often ate the brain and nervous tissue, it made sense that the incidence was highest among women and children. Once the cannibalistic practices stopped, the incidence of kuru dropped and the last recorded death from kuru was in 2005[71,72].

Transmissible mink encephalopathy & Feline spongiform encephalopathy

Farmed mink in Wisconsin began displaying signs of encephalopathy and subsequent work identified it as a novel TSE caused by a prion disease[73]. The feeding of farmed mink products contaminated with scrapie prions was likely the culprit[73] and further outbreaks were likely the result of feeding mink scrapie or BSE contaminated materials. TME is less important as a disease of farmed mink and more important to this work as much of the prion strain research is the result of hamster adapted TME. Similarly, feline spongiform encephalopathy was found in wild and domestic cats, likely after the feeding of BSE-contaminated material[74,75].

Bovine spongiform encephalopathy (BSE)

BSE is a prion disease of cattle that is believed to have arisen from food material either contaminated with scrapie prions or with BSE prions from an animal with atypical BSE (atypical BSE is thought to be a spontaneous disease that naturally occurs at a low rate in aged cattle). The use of meat and bone meal and mechanically recovered meat to create a protein source for cattle is thought to have been the introduction of prions into cattle feed. Subsequently, BSE prions were introduced into the human food chain as beef and sparking the vCJD epidemic in the UK. The significance of BSE is the remarkable ability of the prions to break the species barrier and cause zoonotic transmission events, as is believed to have happened in TME, FSE and vCJD (discussed below)[76,77].

Creutzfeldt-Jakob Disease (CJD)

CJD is another TSE of humans that comes in multiple forms: sporadic, iatrogenic, genetic (i.e., GSS and fatal familial insomnia), and variant. Iatrogenic transmission of CJD occurred when patients had infectious EEG probes used, corneal transplants or growth hormone treatments[78]. Sporadic CJD (sCJD) occurs when, for reasons not yet well understood, there is a spontaneous misfolding of the prion protein that results in disease. In cases of genetic prion disease, patients have a mutation in the *PRNP* sequence that predisposes them to protein misfolding and the development of disease. Finally, and most

interesting, is variant CJD (vCJD), which was the result of a zoonotic transmission of BSE prions to humans by way of contaminated meat, underscoring the necessity of understanding zoonotic prion transmission[76,79,80].

Chronic wasting disease (CWD)

Chronic wasting disease is a TSE affecting captive and free ranging cervids world-wide. It is the only prion disease known to infect free-ranging animals. CWD will be discussed *ad nauseum* below.

Camel prion disease (CPD)

Camel prion disease is the most recent prion disease to be discovered[81]. To date, little is known about CPD. In the first report of CPD, prevalence was low (3.1%) in aged camels (>8 years old). Most camels are slaughtered at a younger age <5, so the prevalence is likely higher than these initial numbers suggest, but the burden of CPD is not yet well understood. Hopefully, CPD doesn't have them same zoonotic characteristics as BSE and won't enter the human food chain.

The cellular prion protein

Discovery of the cellular prion protein being a necessary factor for the development of prion disease that transitioned from primarily alpha helical to beta sheet was critical to our understanding of the disease but begged the question as to the role of PrP and why it was produced in animals. PrP is a highly conserved protein, but what this protein actually does has been a topic of debate. There is research to suggest that it is proapoptotic, antiapoptotic, important in nerve development, copper binding, cell signaling and protective from apoptotic stress, among others[76,82]. Interestingly, PrP knock-out mice are apparently healthy with few differences from PrP-competent mice[76]. There is wide-spread expression of the prion protein, but expression is found most highly in the brain, then followed by the cells of the immune system, most notably follicular dendritic cells, and to a lesser extent on other immune cells[76]. It is likely the role of PrP differs between the brain and the immune cells on which it is also expressed. The quest to determine the homeostatic function of PrP continues…

While the function of the prion protein remains enigmatic, much has been studied about the structure of the cellular prion protein. The prion protein has an unstructured N-terminus followed by an octapeptide repeat, charged cluster, hydrophobic core, followed by a PK resistant core with two glycosylation sites and a single disulfide bridge, and on the C-terminus of the protein there is a GPI-

anchor that keeps the protein located in lipid rafts on the surface of cells[76]. Unlike the diseased form, PrP^{C} is a soluble protein that is PK sensitive and is sensitive to denaturing agents. It is these biochemical differences that facilitates the discrimination between PrP^{C} and PrP^{Sc} [76].

Many ways to misfold: prion strains

One of the most fascinating aspects of prions and is the apparent transmission of unique disease features in the absence of any genetic material – in fact, it was the clear appearance of strains that was often used to argue against the protein-only hypothesis[83]. This epigenetic transfer of information has been, and continues to be, a fascinating aspect of prion diseases. The first report of different strains came from Pattison and Millson in 1961 when scrapie brain was passaged into goats and animals presented with a drowsy or scraping phenotype[19,84]. As early as a 1976 a review by Richard Kimberlin discussed a number of the different strains of mouse-adapted scrapie and some of the differences between the strains and incubation time in mice, how strains were characterized (mostly by neuropathology) and noted that: "…if a group of animals are infected under standard conditions of dose, route of inoculation, strain of agent, and genotype of recipient animal, etc., then there is a remarkable uniformity of response." [85]. This is still the basis on which prion strains are biologically characterized today[86].

Disease presentation and neuropathological lesions were used to characterize prion strains but understanding how a protein could convey epigenetic information and confer strain characteristics remained enigmatic. It was hypothesized that three-dimensional differences in the structure of the infectious prion could instruct the naïve prion proteins to misfold in a similar manner. Much of the early work to understand prion strains was conducted using a newly emergent prion disease: transmissible mink encephalopathy (TME). TME is a prion disease affecting farmed mink and it is thought that the outbreak of TME was the result of feeding the mink scrapie-infected sheep or bovine spongiform encephalopathy (BSE)-infected cattle material to the mink. Passage of TME from an infected mink in WI (origin thought to be bovine) was serially passaged into Syrian golden hamsters and two distinct prion strains emerged: hyper (HY) and drowsy (DY). They HY strain is characterized by hyperexcitability, tremors, incoordination and a short incubation period with widespread infectivity in tissues, whereas the drowsy strain has a much longer incubation period and hamsters present with progressive lethargy. Only CNS tissues from DY infected hamsters harbor infectivity[86,87]. The excellent work of Bessen *et al*. looked

at the prions from infected animals and noted biochemical differences in the features of the two prion strains, providing strong evidence for structural information being able to encode and transmit information between animals[87–89].

Strains are an interesting aspect of prion disease and the evidence clearly indicates that strains are able to be characterized and have a heritable phenotype. However, it is only after serial passage into animals (a process known as strain stabilization) that the extraordinarily stable strain characteristics emerge. Typically, when a strain is stabilized, there is a decrease in incubation and the variability in disease incubation is much smaller than the initial passage. This finding was noted in 1978 when scrapie was passaged into hamsters 6 times. In this study, the brains of earlier passages were also analyzed and a picture of the strain emergence process emerged. They found that on the third passage, two distinct strains were present (431K and 263K) and that these two strains had differing abilities to transmit to mice, with 431K being highly pathogenic. By the 6th passage, only the 263K hamster-adapted scrapie strain was still present. They postulate that the strains they observe in the hamsters is an adaptation of one strain (most pathogenic to hamsters) from a mixture of strains present in the scrapie-infected sheep[90]. The inoculum they used was from “drowsy” goat scrapie → 3 mouse passages → 12 rat passages → 6 hamster passages. Another interesting note in this paper is that two prion strains that looked identical in a mouse (Chandler and 302K) but had remarkable differences when passaged into hamsters.

The results of the experiments by Kimberlin and Walker demonstrate an interesting process known as strain adaptation, where prions from one species are transmitted into another, and then adapt to the host by way of shorter incubation time and consolidated disease features. After inoculation and serial passage, it’s thought that the prions then adapt to their new host; this is a major way in which novel strains have been generated[84,91]. Back to our model HY/DY strain system, HY causes a faster disease in the hamsters and is transmissible, it doesn’t transmit back to mink, but DY causes disease in mink with a relatively short incubation period and complete attack rate[87,91]. These results highlight the significant impact that strain adaptation can have on a prion strain and bring up the importance of intermediate hosts that may enable more facile crossing of the species barrier or changing of host ranges altogether[84,91].

A major confounding factor here is that the prion protein being input is different than the PrP structure of the recipient animal. This is known as the species barrier. It is generally accepted that

differences in the sequence and structure of the prion protein can impede or entirely impair the ability of the prion to replicate and may result in the emergence of a novel strain. The development of numerous transgenic mouse lines that express species-specific prion proteins have been instrumental in strain-typing studies and, by removing the PrP species barrier, enabling effective study of prion strains without the need for cross-species adaptation. While transgenic mice have greatly aided the study of prion strains, strain emergence can be altered by non-host factors[92] or gene dosage in transgenic mice[93], complicating the interpretation of the data on strains after passage into model, transgenic animals and highlighting the possibility of artifacts being introduced into strain typing models. This is an important motivator for the research conducted in this dissertation.

While strain adaptation and potential confounding influences of models complicates the understanding of strain emergence, there is also evidence of prion strain maintenance in animals without adaptation. More specifically, Bian *et al.* demonstrated that prions from one species are able to replicate within certain transgenic mice with PrP of a different species (e.g., horse), but retain the infectivity and strain characteristics of the input strain and do not adapt to the recipient host[94]. Termed nonadaptive prion amplification (NAPA), this work has challenged many assumptions about strain adaptation and provides interesting hypotheses about the species barrier perhaps being more reliant upon incoming PrP^{Sc} species rather than solely the recipient PrP^{C} structure. In their discussion, they highlight how NAPA may provide an explanation for epidemiological results seen in the vCJD outbreak[94].

Strain interference

Prion strains have also been shown to interfere with one another to delay infection or cause an intermediate phenotype of disease. As early as 1975, Dickson and colleagues inoculated mice with 22A and then superinfected the mice with 22C. Both were mouse-adapted scrapie strains, where 22A had a long incubation period (~500 days) and 22C had a shorter incubation time (~230 days). 22C was unable to cause disease in the animals if they had previously been infected with 22A prions, but this wasn't the case when mice were first inoculated with normal mouse brain. This was the first evidence of strain interreference and competition[38,85]. Later, Bartz demonstrated similar findings with hyper superinfection of drowsy-infected hamsters while also demonstrating the necessity of the same neurons being infected to have the interference effect[95,96]. Interestingly, it appears that the blocking strain appears to sequester

PrP^C from the superinfecting strain[97]. These results were also shown with natural isolates by an oral route, highlighting the possibility of this occurring in nature[86,98].

What influences prion strains?

While much of this early work with strains involved breaking species barriers and strain adaptation resulting in a vast number of mouse-adapted scrapie prion strains, more recent work has demonstrated other important factors that dictate strain emergence and properties. Perhaps not surprisingly, PRNP genotype can have an important role in prion strains. For example, this is evident in sheep infected with scrapie[66], humans with various forms of CJD, and CWD (discussed below). Interestingly, sheep that have a genotype resistant to traditional scrapie (ARR) are able to be infected with a novel scrapie strain, Nor98, that doesn't transmit to sheep susceptible to classical scrapie (ARQ)[66]. In human CJD, a polymorphism at codon 129 dictates susceptibility (M) or resistance to disease (V) with different disease presentations depending upon the genotype of the affected individual[99]. Six types of sCJD are recognized depending on biochemical features and host genotype[100].

In addition to genotype, cellular cofactors can play a role in the emergence of prion strains. Recently, a cellular cofactor, phosphatidylehanolamine (PE), was shown to enhance *in vitro* PrP^{Sc} conversion and, more interesting, when PE was included in protein misfolding cyclic amplification, three distinct mouse strains used to seed the reaction all converged in the presence of PE and looked like a single strain when propagated into animals[86,101]. Additional work with cofactors demonstrated that RNA and palmitoyl-oleoyl-phosphatidylglycerol (POPG) influenced the folding of the prion protein[102] and other work has shown that removal of RNA during PMCA, then reintroducing it to the reaction, results in dramatically different prion strain properties than the original inoculum[103,104]. These studies support that cellular cofactors and/or the cellular environment may be influencing strain emergence and presentation. Additional work supports that host factors other than genotype can influence prion strain diversity[92]. How the presence or absence of these cellular cofactors are functioning within an infected animal and/or differences between a natural host and model organism remains to be seen. Post-translational modifications may also be important in prion strains. Specific recruitment of sialoglycoforms has recently been shown to dictate strain-specific structure[105,106]. There also appears to be a role in the immune

system for selection of different prion strains, with different disease presentations in mice lacking complement regulatory protein factor H (fH)[107].

How could new prion strains emerge?

While there appear to be many contenders for controlling strain properties and strain emergence, where new strains could come from is still a matter of debate. Under the prion "cloud" hypothesis, PrP^{Sc} isoforms exist within a host, with one dominant species giving rise to the features seen in disease, but multiple sub-strains exist within the host and replicate at a lower level[108]. This idea is akin to quasi-species in virology. Another hypothesis is deformed templating being able to give rise to novel prion strains[109]. Under deformed templating hypothesis, it is thought that prion species that interact with PrP^{C} that is slightly different will have a deformed template, and possibly give rise to novel PrP^{Sc} strains. Both these hypotheses are likely true and prion strains likely emerge from both a "cloud" of prions from a host and as a result deformed templating when crossing the species barrier or interacting with a novel genotype

Even more interestingly, *de novo* generation of a prion strain with distinct biological and biochemical features has been demonstrated in PMCA[110]. These data, along with the clinical findings with sCJD and cofactor experiments, suggest that the prion protein can misfold into multiple conformations spontaneously (perhaps dependent on the cellular context) and give rise to novel prion strains. This possibility, in combination with the aforementioned cloud and deformed templating hypothesis, present a third way in which a novel strain or substrain could emerge.

How are strains defined?

Prion strains are difficult to define because there is still much debate about the degree of difference required to call something a novel strain. Operationally, prion strains are defined as a disease presentation under a fixed set of host and agent parameters[84,86,91]. In the lab, strains are characterized by a number of biological features observed upon passage of an infectious prion into a model organism. Specifically, clinical disease presentation, incubation time and histopathological lesions and utilized to characterize prion strains. Biochemical features such as electrophoretic mobility, glycosylation pattern/glycoform ratio, proteolytic resistance and resistance to denaturation (i.e., conformational stability) are also used to characterize the prion strain. A key feature of prion strains is their heritability upon serial

passage. Prion strains are excellently reviewed in Morales *et al.* 2007[84]; Morales 2017[91]; and Bartz 2016[86]. This method is as old as prion research, but remains tried and true, despite the extraordinary cost of animal bioassay. Collections from prion infected animals that have not yet been serially passaged in mouse models are more aptly called “isolates” rather than strains because the sample has not yet been put through the rigorous bioassay to fully characterize the strain.

Prion “immunology”

Prion diseases do not elicit an immune response because of immune tolerance of self-proteins[66,111–114]. While prions do not generate a typical immune response, prions have significant interactions with the immune system. Indeed, as early as 1978 it was noted that a loss of spleen resulted in delayed neuroinvasion[115]. In natural systems, oral exposure to prions is believed to be the primary route of prion infection, including vCJD, BSE, scrapie, TME and CWD. In many prion diseases, there is a profound lymphtropism and early replication of prions in secondary lymphoid organs prion to neuroinvasion. After oral ingestion, M cells in the small intestine are important in the uptake of prions [66,111–114]. Indeed, an increase in M cells has been shown to accelerate the onset of prion disease[111] and a proinflammatory state in the gut either by bacterial[116] or parasitic infection[117] also potentiates prion disease, likely in the small intestine as large intestine-restricted parasites did not exacerbate disease[112].

After uptake of prions by M cells into Peyer’s patches, prion replication occurs in secondary lymphoid organs. The complement system plays an important role in prion pathogenesis and complement proteins C3[118,119], C1q[119–121], fH[107] and complement receptor CD21/35[122–124] are important for potentiating prion pathogenesis[125]. While many complement proteins are important, other complement proteins, such as C5, have been shown to not be important in prion pathogenesis[126]. The complement receptor CD21/35 has been shown to bind tightly to prions[124] and the loss of CD21/35 is highly protective to prion disease[122,123]. Because the absence of CD21/35 is so protective against peripheral prion accumulation and ultimate neurological disease, it has been proposed as a prion receptor that may select for specific prion quasispecies[127]. From the adaptive immune system, B cells and T cells both express PrP^C, but only B cells have been shown to play a role in peripheral prion pathogenesis, perhaps by supporting FDCs[127] or by intranodal prion trafficking[128].

While many immune cells express PrP^C, it is follicular dendritic cells (FDCs) that are requisite for extraneural prion amplification. The binding of prions to many complement proteins and complement receptors is necessary for the amplification of prions in secondary lymphoid organs[127,129]. FDCs typically capture and present antigens for long periods of time have been shown to be required for prion amplification[130]. FDCs have receptors for complement-bound antigens and likely bind to prions in a complement-receptor mediated manner. FDCs are also likely important for neuroinvasion and studies where the distance between FDCs and sympathetic neurons was decreased, prion disease was accelerated[130].

Lymphoid prions were shown to have increased zoonotic potential than brain-derived prions from the same animal[131] and some differences were found between brain and lymphoid prions from deer after bioassay in transgenic mice[132]. These results, along with the hypothesis that CD21/35 and perhaps other complement proteins are selecting for specific strains to propagate a small proportion of neuroinvasive prions, implicate secondary lymphoid organs as potential sites for expansion of prion quasi species. This has important implications for cross species transmission as it is not typically brain material that is consumed by humans and differences between brain and lymphoid-derived prions warrants further study to best assess the risk of zoonotic prion transmission.

The most interesting one: Chronic wasting disease

Here we go again: discovery and spread of a novel TSE

Chronic wasting disease was first identified in captive mule deer (*Odocoileus hemionus*) in Colorado in 1967 and was first reported in the literature by Williams and Young in 1980[133]. The new, curious disease presented with listlessness, polydipsia, polyuria, excessive salivation, teeth griding, drooping of the head, strange behavior with a lack of fear of humans and fewer interactions with other heard members, occasional hyperexcitability and progressive weight loss[133]. Pathologically, the deer had spongiosis in the brain and reactive astrocytes, hallmarks of spongiform encephalopathies. Just two years later, a spongiform encephalopathy of Rocky Mountain elk (*Cervus canadensis nelsoni*) was also reported[134]. The affected elk had fence line contact with the affected deer and were housed in pens that had previously held infected deer and the disease was likely transmitted by contact with infected mule deer/paddocks. Elk in Colorado and Wyoming presented with many of the same symptoms as the mule

deer, including a drooping head, excessive salivation and wasting. Immunoreactivity and staining of CWD amyloid plaques with anti-scrapie antibodies[135], additional histopathological findings[136] and identification of fibrils in the brains of CWD-infected animals by EM[137] would solidify the placement of CWD among the transmissible spongiform encephalopathies. In 1997, the first cases of CWD would be reported in free-ranging mule deer, white tailed deer and elk in northern Colroado[138]. An infected elk that was transported from Canada to South Korea was the first reported case of CWD outside of North Ameria[139] and future characterization work would indicate the strain was that of classical, North American CWD[140]. In 2001, cases in free-ranging white-tailed deer were reported in Wisconsin and were a notable expansion of the range of CWD[141]. A free ranging moose (*Alces alces*) was diagnosed with CWD for the first time in 2007[142]. The explosion of CWD continued from there and widespread across the US and Canada (Figure 1.1), South Korea and, in 2016, Europe found its first cases of CWD in reindeer[143].

The list of susceptible cervid species is staggering and seems to get longer with every cervid species examined. Thus far, white-tailed deer (*Odocoileus virginianus)*, mule deer, black-tailed deer (*Odocoileus hemionus columbianus)*, elk, moose, red deer (*Cervus elaphus*)[144,145], reindeer (*Ranfiger tarandus tarandus*)[143,146], fallow deer (*Dama dama*)[147] and sika deer (*Cervus nippon*)[148] have either been infected with CWD naturally or been susceptible upon experimental transmission (fallow and Sika). Free ranging populations of sika deer have fortunately remained CWD-free to date[149].

From deer to deer: CWD transmission

The explosion of CWD was unlike any other and, to date, is the only prion disease that is known to affect both captive and free ranging animals (Figure 1.1). Understanding how the disease was transmitted was paramount to getting a handle on the disease. Studies of the captive mule deer populations found that horizontal transmission was the most important to the maintenance of CWD[150]. Subsequent studies have found that infectious prions are found in the brain and lymphoid tissues, blood and saliva[151], antler velvet[152], skeletal muscle[153], urine[154], feces[155,156], nasal brushings[157], fat[158], semen and male reproductive tissue[159], female reproductive tissues[160] and in the pregnancy microenvironment[160–162]. While horizontal transmission has been demonstrated to be essential to CWD maintenance and transmission, there is evidence of maternal transmission of CWD that may also play a role in CWD transmission, perhaps by both vertical transmission to fawns *in utero* as well as introduction

of infectious birthing materials into the environment[162–164]. Unfortunately for cervids, a very small amount of infectious material is able to cause infection[165].

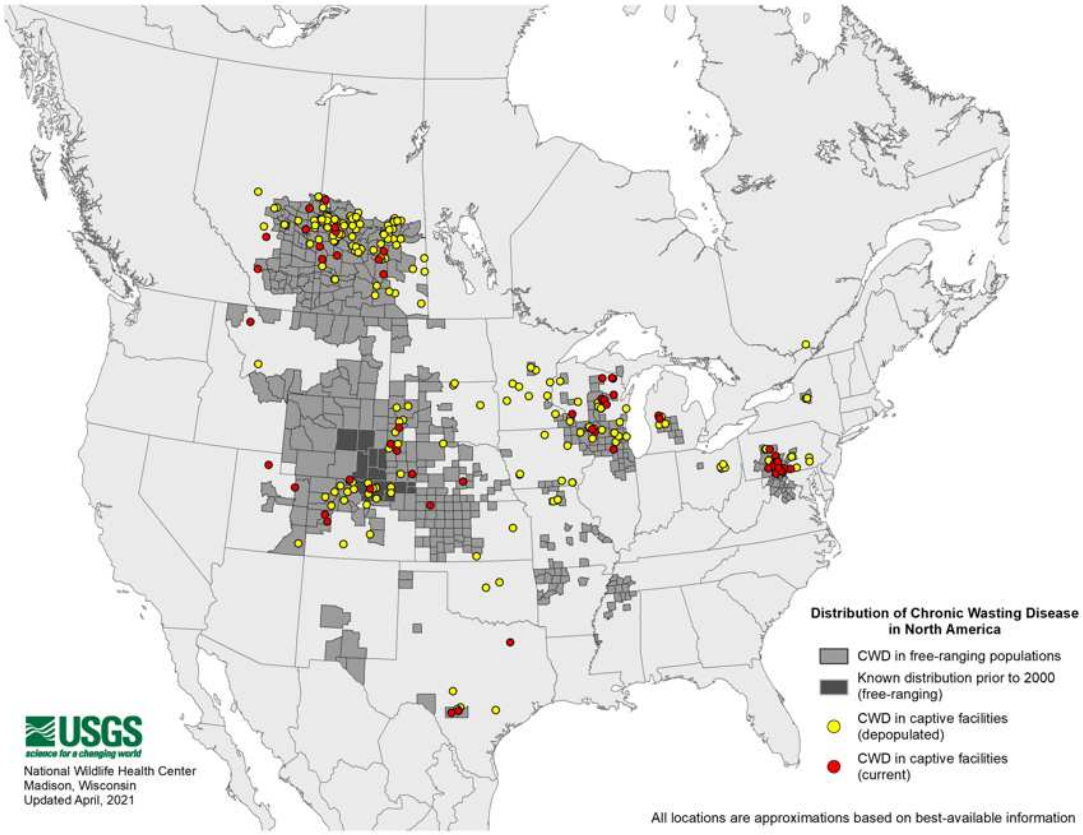

Figure 1.1 Distribution of CWD cases in North America. Image from USGS.

Clearly, infected deer shed prions from a multitude of sites once infected. When a susceptible animal comes into contact with prions, there is early replication in lymphoid tissue as CWD is profoundly lymphotrophic[129,166] with PrP^{Sc} in lymphoid tissues of preclinical deer[167]. Studies of the timeline of CWD infection in animals demonstrates early lymphoid replication by 4 months post exposure at levels usually observed in terminal disease[168] and infectious prions in feces are detected 6 months post exposure[169]. There is also positivity in the nerves that innervate the gut, highlighting a possible route of neuroinvasion[170]. This early, preclinical, lymphoid replication and shedding is believed to be one of the reasons why CWD is so transmissible among cervid populations[171].

Direct contact between infectious and susceptible cervids is thought to be the primary route of CWD transmission, but infections of animals that were only exposed through environmental contamination or fomites indicated that there could be environmental transmission of CWD[167,172]. Similar observations were made in cervidized mice infected with CWD: mice shed infectious prions in their feces[156] and were able to be infected by way of soil contaminated with prions[173]. Scrapie, which also can be transmitted horizontally, was found to still be infectious 16 years after eradication of an infected flock of sheep, suggesting that stability of prions in the environment is exceptionally long[70]. Together, this evidence and mathematical models were highly suggestive of the potential for environmental prion transmission.

The danger in the dirt: environmental prion transmission

As mentioned, prions are shed from preclinical and clinical deer and prions are found ubiquitously in infected animals exemplifying a multitude of ways prions can be introduced into the environment. Soil plays a critical role in CWD transmission. Specifically, Montmorillonite clay (Mte) is a common clay component of soils that binds to prions. Interestingly, when prions are bound to Mte, they have increased bioavailability[173,174], likely because M cells in the gut are more likely to uptake Mte-adsorbed prions. Additionally, a 1% increase in the size of clay components in soil was associated with a nearly 9% increase in the risk of an animal having CWD, highlighting how soil components may be important in dictating CWD infections[175] (but see comment in Saunder *et al*.[176]).

While adsorption to clay increases bioavailability and is associated with increased risk of CWD, there is some hope for natural prion degradation. Soil-bound prions were shown to be more readily inactivated than unbound prions under simulated cycles of drying and wetting, suggesting bound prions may inactivate more readily in nature[177]. Soil organic matter, specifically humic acid, was shown to reduce recovery and infectivity of CWD[178]. More recent work did have some unfortunate findings for the deer. After testing prions with many additional soil types and components, Kuznetsova *et al.* demonstrated decreased prion recovery and detection over time (except for quartz), but despite decreased detection, the samples remained infectious[179]. While very interesting, it is unfortunate that prions are unable to be detected but remain infectious because it will complicate environmental monitoring of prions[179]. Finally,

lichens have also been shown to have prion-degrading capabilities and can maybe reduce the environmental prion burden[180].

Soil is consumed intentionally for mineral content and inadvertently while grazing on plants, providing a realistic route by which soil-bound prions can infect a naïve animal[175,181]. Additional work demonstrated that soil binding was slower and reduced when brain homogenate was used for binding to soils[182] and found that for prion strain interreference properties were altered under simulated natural conditions using HY and DY[183], highlighting the importance of understand strain-specific soil/prion interactions and the use of relevant sources of prions to fully understand the environment/prion interactions.

In addition to soil, water from CWD-endemic areas had evidence of infectious prions[184]. Plants have also been shown to bind to prions on their external root structures[185], uptake prions when root systems were damaged[186], most recent work has shown that infectious prions bind to external surfaces and uptake prions from soil into aerial tissues[187]. As if the poor deer didn't have it bad enough already, coyotes[188] and crows[189] are able to disseminate infectious prions through their digestive systems across the landscape without becoming infected themselves. This may be a route by which new cervid populations can become exposed to CWD. Perhaps there are yet undescribed instances of NAPA occurring that are also exacerbating the prion burden on the landscape[181].

Deer behavior and deer death

Cervids often congregate in areas during the winter, creating ideal conditions for animal-to-animal transmission and high prion burdens in the environment[150,190]. Salt licks have also been shown to congregate deer and act as environmental reservoirs[191]. Prime-aged mule deer males generally have a higher incidence of CWD[190], but white-tailed populations have found higher prevalence in females[192]. Female white-tailed deer form matrilineal groups and spend a lot of time in the same area and groom each other, explaining the high prevalence among female white-tailed deer[193]. Behavior also plays an important role, with male cervids participating in rub-urination (particularly during mating season), fighting with other males and interacting with as many females as possible[194]. Urinating on oneself and interacting with other male and females is likely posing a significant risk factor for CWD transmission, especially during the rut. While direct contact is an important part of CWD transmission, there is evidence of infected

animals interacting less with other animals when suffering from clinical CWD, but the preclinical shedding of CWD suggests that this isolation may not mitigate transmission much.

Logically, deer with CWD are more likely to die than uninfected deer[195]. Mountain lions selectively prey on CWD-infected deer and highlight the importance of considering predator-prey interactions for CWD management[196,197]. While not statistically significant, deer with CWD tended to be more likely to be involved in vehicle collisions, implying cognitive impairment in pre- and clinical deer[198].

What about the genotype?

Similar to other prion diseases, genotypes conferring susceptibility and resistance in multiple cervid species have been found (Table 1.1) and reviewed[199]. Of note, in free-ranging elk populations with CWD, there appears to be selection for CWD-resistant genotypes[200]

Table 1.1. Susceptible and resistant genotypes of commonly CWD-infected cervids.

	Species			
Genotype	White-tailed deer[201–203]	Mule deer[204]	Elk[205]	Norwegian Reindeer[206,207]
Susceptible	96GG	225SS	132MM	225SS
Resistant	96SS	225FF	132LL	

Save the deer! CWD management and mitigation strategies

The future of cervid health appears bleak after the discovery of CWD. Multiple mathematical models suggest that CWD will have negative population level effects in the near future and suggest that culling effects will become less effective over time because of the long environmental incubation of prion diseases[192,208,209]. Because of the insidious nature of the disease and the devastating potential of long-term infection in populations, many state/government agencies have taken CWD control very seriously.

New York is an example of a success story in the control of CWD. In 2005, the first case of CWD was found in New York state in two captive white-tailed deer herds; the captive populations were depopulated quickly. Troublingly, one of the populations was attached to a taxidermy studio that was also functioning as a white-tailed deer rehabilitation center. Immediately, a 10-mile radius containment zone was instituted, and when checking for CWD positivity, 2 wild deer were positive for CWD. Upon finding

positives in wild deer, emergency mandates were put into place to prevent further disease transmission, including mandatory hunter check points, strict movement bans on carcasses and other cervid material, and extensive taxidermy record keeping. The measures proved to be effective – after testing >7,000 animals between 2005 and 2010 with no CWD cases, the containment zone was decommissioned and, to date, no more cases of CWD have been observed in New York. To date, this is the only success story of eradicating CWD[210].

The story of CWD in Wisconsin and Illinois is an interesting case study in the importance of government culling and CWD management and will be discussed here. In 2002, there was an outbreak of CWD on the WI-IL border. Both states used government-sponsored sharp shooters between 2003 and 2007. Political and public pressure in WI resulted in the end of the program, but sharpshooting continued in IL. An analysis of CWD positivity 5 years later found that, on average, CWD in WI increased 0.63% each year after ceasing the sharpshooting programs, whereas CWD rates in IL did not change over the same time period[211]. The situation in New York, Wisconsin and Illinois highlight the successes, failures and necessity of managing cervid populations to control CWD.

In 2016, Norway reported the first case of CWD in their reindeer population[143]. With no known connection back to the US, the discovery of CWD was surprising and disheartening. In 2018, a novel type of CWD was detected in a Norway moose[212] and a recent report from Sweden found that 3 aged, female moose had similar CWD with little lymphatic involvement[213]. This CWD presentation in these moose is believed to be of spontaneous origin because of the surprising lack of lymphatic involvement and isn't thought to be as great of a risk to cervid populations. For this reason, moose CWD hasn't been as aggressively managed. Norway has tried to get a handle on the CWD situation in the reindeer in a dramatic way. To prevent further CWD transmission and potentially devastating consequences, difficult decisions were made to eradicate the entire Nordfjella reindeer herd, which comprised of nearly 7% of the total reindeer population and quarantine the environment for 5 years before allowing other animals to enter the area[214,215]. In total, 2,024 reindeer have been culled[215]. Hopefully, the disease was discovered early enough and the measures taken will be sufficient to eliminate CWD from Europe.

In addition to population management strategies, people have attempted to develop a vaccine against CWD. Challenges with vaccine development against prion disease and a summary of attempted

vaccine platforms are reviewed in Zabel & Avery[216] and Aguzzi *et al*. [217], respectively. Specifically, for CWD, Goñi *et al.* showed delayed disease in white-tailed deer (~300-day delay) using a mucosal vaccine against CWD[218]. In another vaccine attempt, Wood *et al*. created a vaccine targeted against a PrP^{Sc} specific epitope and it actually accelerated the onset of prion disease in naturally exposed elk[219]. Development of a vaccine to protect farmed/captive deer would be a substantial step towards control of CWD, but will be a significant challenge to engineer.

It's not all the same: CWD strains

There is evidence for multiple CWD strains. A seminal paper by Angers *et al.* found two distinct strains of CWD (CWD-1 and CWD-2) circulating in North American cervids[220]. CWD-1 had a shorter incubation time and bilateral distribution in brains of infected animals and CWD-2 had a longer incubation time and only unilateral distribution of PrP^{Sc}. Important to the goals of this dissertation, there were no differences in biochemical properties between the two strains. They also found unstable propagation in mice expressing deer PrP, but not in elk PrP, suggesting that there was a conformational dependency for strain propagation. Deer and elk PrP differ by one amino acid in residue 226, implying that residue 226 is important to CWD emergence and strain characterization. Subsequent work built on these observations and found that gene targeted mice expressing deer PrP (226Q) had more disorganized staining patterns and asymmetrical distribution in the brains of infected mice, regardless of inoculum genotype[221]. Taken together, these studies identified two prion strains with unstable propagation in the presence of deer PrP. Importantly, highlights the necessity to continue studies of sympatric cervid species to understand how CWD prions from one cervid species infecting another might influence CWD strain emergence.

In a study to examine the influence of white-tailed deer genotype on strain emergence, Velásquez *et al.* generated a novel strain (dubbed H95+) after passaging the brain homogenate from a white-tailed deer (H95 S95) infected with CWD into tg60 mice (which over express 96SS PrP) and a novel strain emerged after serial passage[222]. The other strain that was found in the study was dubbed Wisc-1. They postulate that Wisc-1 is the same strain as CWD-1, but because different transgenic mouse lines were used the results can't be directly compared[222]. CWD isolates are typically not transmissible to wild-type mice, but the H95+ strain caused disease in wild-type mice[223]. This work highlights the impact that

genotype can have on emerging CWD strains and indicate that these newly emerging strains may have increased cross species and/or zoonotic potential[223].

More recently, research from the McKenzie lab has demonstrated an important influence of host genotype on novel strain emergence in white-tailed deer CWD. In this study, deer of a number of genotypes with polymorphisms at codons 95 and 96 were experimentally inoculated with the Wisc-1 strain of CWD. Brain homogenate from the infected deer of disparate genotypes were serially passaged into either Tg33 or Tg60 mice to monitor for strain emergence. They showed that, depending on the genotype of the mouse, there was emergence of H95 or Wisc-1 or co-propagation of both strains, suggesting that there is a mixture of strains in the inoculum or deformed templating influencing the outcome of the strain upon passage into mice of different genotypes. Importantly, this work has implications for how CWD strains could emerge naturally from cervid species within a herd that express different genotypes and provide evidence that CWD strains are continuously evolving in populations with individuals of different genotypes[224]. The discovery that CWD is resulting in selection of resistant elk genotypes[200] highlights how CWD can change gene frequency, which may have important consequences for strain emergence. It is possible gene frequency changes will also be seen in white-tailed deer populations and this change may influence strain emergence. Furthermore, while not yet published under peer review, preliminary research from the Soto lab suggests the CWD isolates amplified with 96SS substrate had enhanced ability to cross species barriers[225]. Taken together, this research highlights the importance of: 1) monitoring cervid genotype frequencies, 2) continued assessment of CWD strain emergence and 3) continued vigilance of the risk of CWD to humans.

The outbreak of seemingly classical CWD in Norway with no connection back to the US or any other location understanding if this was a novel strain of CWD was of utmost importance. When isolates form Norway were passaged into bank voles, the strain characteristics were different from that of classical, North American CWD[226]. This instance of CWD might represent a new emergence of CWD in a new population. Importantly, when reindeer and moose isolates were passaged into mice expressing human PrP, there was no indication of zoonotic transmission[227].

A novel strain of CWD was generated *de novo* after several rounds of PMCA, highlighting that novel CWD cases may arise from spontaneous cases as well[110]. As discussed in Meyerett-Reid *et al*., the

unique features of the rigid loop of cervid PrP may predispose it to spontaneously misfold and perhaps weaken species barrier as CWD changes over time[110]. These data, combined with the data presented above, highlight the significance of the genotype of the infected cervid on CWD emergence and demonstrate how disparate, novel CWD strains may have different zoonotic ability. It is imperative that CWD from cervids continue to be studied to better understand transmission within and between cervid species, as well as continued assessment of the risk of CWD to human health.

CWD in humans: the coming zombie apocalypse?

In light of the vCJD outbreak that occurred as a result of the BSE outbreak, a lot of research activity has been directed at studying the potential of CWD to cross the species barrier and cause disease in humans. Notably, non-human primate studies have found that squirrel monkeys are susceptible to CWD[228], but Cynomolgus Macaques are not[229]. A widely touted study from Canada has claimed they have demonstrated transmission of CWD to Macaques, but while there has been a flurry of news media about this study, it has not yet been published under peer review. Transmission studies to mice expressing human PrP have failed to cause disease to date in the published literature[227,230,231]. However, *in vitro* studies have shown propensity for cervid prions to misfold human PrP[232,233]. Unfortunately, experimental studies on the cross-species transmission of CWD have not provided a clear answer as to the risk of zoonotic CWD transmission.

Results from case histories and risk associations have also been mixed. In humans, a risk association study between areas that had high CWD prevalence were analyzed for increased risk of development of CJD, but no increased risk was observed[234]. While there wasn't an increased risk of having CJD in those counties, the confounding factors of incubation time and possible aberrant disease presentation cannot rule out the possibility of cross species transmission. In an unfortunate incident, a group of hunters ate venison from an animal that was later determined to be CWD. Many of these individuals have agreed to be monitored for signs of CJD. The most recent report from 2014 was a 6 year follow up on these individuals with no signs of CJD[235]. Retrospective analysis of CJD case files found three cases of CJD in unusually young patients (not unusually young for vCJD, however) that all had a history of consuming venison, suggesting that these were perhaps some of the first cases of zoonotic CWD[236]. Most recently, a 61-year-old patient presented with progressive confusion and gain instability

and Corticobasal Syndrome (CBS) with unusually rapid onset was suspected. After learning the patient had been taking deer antler supplements, CJD was considered as an alternative diagnosis. CSF RT-QuIC and MRI concluded that the patient did indeed have CJD[237].

Could it be possible that zoonotic transmission of CWD is occurring/already has occurred but went unrecognized because the clinical presentation so closely resembled another disease like CBS? Much of the work that has been done with prions and prion strain typing have involved brain-derived prions, but the potential increased zoonotic transmission of extraneural prions warrants additional studies on peripheral prion strains[131]. There is no clear evidence that CWD causes disease in humans, but the possibility cannot be excluded. Vigilance for zoonotic transmission and understanding the diversity and distribution of CWD strains and their potential for zoonotic transmission, are of the utmost importance.

Introduction to the Work in this Dissertation

The goal of this research was to characterize prion strains from a number of free-ranging animals by looking at primary isolates from the natural host characterize the diversity and distribution of CWD both within and between deer, as well as add to our understanding of plant-vectored CWD transmission. To address strain diversity, we procured lymph node samples from collaborators across the country and assessed prion strain characteristics by conformational stability, electrophoretic mobility, and glycoform ratio. Further, we assessed strain differences within a host between two tissues using the same methods. To assess the possibility of CWD transmission by a plant vector, plants were exposed to CWD prions and PMCA was utilized to detect low levels of prions. The research shown here provides evidence of prion strain differences between tissues, locations, showcases ways in which CWD can present in surprising ways and adds to a body of work implicating plants as a short-term vector of CWD prions. This work has important implications in our understanding of CWD strains in a natural host and plant-vectored transmission.

Aim I: Assess prion strain differences between lymph node and obex samples within a single host

The goal of this aim was to determine if disparate prion strains were found when comparing lymph node and brain tissues within the same animal. Because of the cost and pitfalls of relying so heavily on mouse bioassay, we utilized biochemical strain typing techniques to elucidate strain

differences biochemically. Based on reports of increased zoonotic potential of extraneural prions, we hypothesized that there would be greater diversity of prion strains within lymph nodes than in the brain. Utilizing biochemical strain typing techniques, we show that there is more variability in lymph node isolates, implicating more strain diversity, than in the brain. This work has important implications as to how we study prion strains.

Aim II: Characterize biochemical prion strain variability within and between outbreaks in the US

There are two hypotheses for the origin of CWD: 1) a cross species transmission of scrapie into cervids caused the first case of CWD that subsequently spread worldwide and 2) CWD originated from a spontaneous conversion of PrP in an animal in Northern Colorado that was then transmissible between cervids. If the former is true, all CWD cases originated from a single case, but if the later is true, there could be numerous strains of CWD from instances of spontaneous conversion that sparked different outbreaks. Understanding the strain distribution is important to elucidate the origin of CWD. Generally, CWD is considered a homogenous disease, but recent reports of novel strains causing geographically distant outbreaks begs the question how many outbreaks are caused by novel strains of CWD. By characterizing strains from numerous CWD isolates from both contiguous and noncontiguous outbreaks we hoped glean insights as to the distribution, origin and evolution of CWD in natural isolates. We hypothesized that we would find a greater diversity of prion strains than is currently documented in the literature. In chapter 3 we show that while CWD is, overall, quite similar biochemically, there are specific CWD isolates and within-state CWD clusters that suggest the presence of a novel strains that warrant follow up investigation. This work provides important insights as to the distribution and occurrence of strain diversity and highlights specific animals/locations that should be followed up with by state agencies or bioassay.

Aim III: Characterize an isolate from TX with novel biochemical properties to determine if it is a novel strain and assess zoonotic transmission risk to humans

During our biochemical strain typing project, two isolates from farmed deer in TX were submitted. Upon analysis of these samples, it became immediately apparent that these samples were, biochemically, very different from the other samples submitted for strain typing analysis. Because of the unique presentation of these isolates and the knowledge that there was no known connection of these

CWD cases to other cases, we hypothesized that these isolates represented a novel strain of CWD with unusual biochemical characteristics. After bioassay, these isolates present as typical CWD in Tg33 mice and there is no evidence of increased risk of zoonotic transmission. Interestingly, these results show that CWD can present in a novel way when we assess samples directly from a native host, but upon passage into a mouse models, presents in a typical fashion. Importantly, this work highlights the differences in prion strains characteristics from a natural host.

Aim IV: Determine the role of plants in CWD transmission

To control CWD in cervid species, it is imperative to continue the assessment of possible transmission routes. Previous work has proposed that plants are able to uptake and transmit infectious prions. We continued to investigate the potential role of plants in CWD transmission. We hypothesized that another plant species, rice plants, would also be able to uptake infectious prions shortly after exposure in a hydroponic system. Interestingly, we found that plants had infectious prions shortly after infection, but apparently cleared the infection by 72 hours post infection. These results imply that different plant species have differential ability to uptake and maintain infectious prions and will be informative to assessing the risk of vectored CWD transmission.

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Chapter 2:

Biochemical differences in chronic wasting disease prion isolates from the brain and lymph nodes of free ranging, naturally exposed white tail deer

Summary

Chronic wasting disease (CWD) is an invariably fatal prion disease affecting cervid species world-wide. CWD is profoundly lymphotrophic and most of the prions that are deposited from the animal are from peripheral sites, however, CWD is still ultimately a neurodegenerative disease. For this reason, much of the research that has been done has focused on brain-derived prions. There is a significant knowledge gap for understanding if brain-derived and lymph-node derived samples are composed of prions of similar structures. There is also research demonstrating the increased zoonotic potential of extraneural prions, perhaps because peripheral and CNS prions exist as different structurally different prion strains. The purpose of this research presented here was to examine prions from the obex and lymph node of naturally exposed white-tailed deer to determine if any biochemical strain differences could be identified. We found biochemical evidence of strain differences between the brain and lymph node from these animals. Future work should examine the biological impact of these biochemical differences and examine more cervids from multiple locations to see if these differences are conserved across species and locations.

Introduction

Chronic wasting disease (CWD) is unique among prion disease as it is the only prion disease that is known to infect and be naturally transmitted between both captive and free-ranging populations. Mathematical models indicate that horizontal transmission of CWD is the most prevalent form of transmission[1–6], but vertical transmission has also been demonstrated and also contributes to CWD transmission[7–9]. Infectious prions have been detected in excreted bodily fluids including saliva, urine and feces, as well as antler velvet, blood and reproductive tissues[7,10–13]. While CWD causes a neurological disease, it is a profoundly lymphotropic disease and these peripheral prions are the most likely shed into the environment and contributing to horizontal and vertical disease transmission[14–18]. Thus, it is critical to

determine if there are any unique characteristics of extraneural prions that affect CWD pathogenesis and transmission.

While all prion diseases result from a misfolding of the normal host protein, PrP^{C}, to a misfolded form, PrP^{Sc}, different biochemical characteristics and disease phenotypes suggest a phenomenon of prion strains transmitting unique disease characteristics epigenetically enciphered within unique prion structures[19–21]. Thus, different prion strains sometimes have significant differences in disease presentation, transmission and zoonotic potential. There are multiple CWD strains that have been identified from North American white-tailed deer, including CWD-1, CWD-2, H95+ and Wisc-1[22,23]. Understanding strain differences and potentially different transmission dynamics is of critical importance to our understanding of and ability to control CWD.

Of particular concern, extraneural prions have been shown to have increased zoonotic potential [24] and the H95 prions strain has also shown evidence of increased ability to cross species barriers[23]. This has important implications for cross species transmission and risk for humans potentially contracting CWD from eating infected skeletal muscle or while cleaning a deer in the field[25]. While there is currently no evidence of CWD prions naturally infecting other conspecific, non-cervid, animals, CWD prions are infectious to cattle[26], sheep[27], swine[28], and cats[29] when inoculated intracerebrally. Importantly, cattle and cats were resistant to CWD infection after oral exposure, but pigs were susceptible at low levels[28]. These data suggest that there is some risk of transmission to additional species and populations in the future, warranting continued monitoring and surveillance of CWD prions and strains.

Much of the CWD and prion research that has been completed to date has focused on brain-derived prions, likely because prion diseases are neurodegenerative, but also because brain samples are easy to work with and contain the highest titers of prions in infected animals. However, prions shed into the environment are likely to be extraneural prions, such as found in lymph nodes. Far less is known about the transmissibility and structure of these peripheral prions. Furthermore, numerous immune receptors and different proteins involved in the complement cascade have been shown to influence prion strain selection, implicating the immune system as an important player in prion strain selection[30–36]. This research suggests that lymphogenic prions likely exhibit more strain diversity than neurogenic prions [37,38]. Tissue-specific differences in strain heterogeneity, as reflected in the prion cloud hypothesis, predict

different prion strains with different biochemical and structural characteristics, in lymph nodes than in the brain. Therefore, any differences between the brain-derived and lymph node-derived prions must be investigated to aid our understanding of intra-host and inter-species prion dynamics.

Based on what we know about CWD transmission, prion strain selection and differential zoonotic transmission, we hypothesized that there are different and more diverse CWD strains in the lymph node than in the brain from the same individual animal. We also predicted that this would be consistent across multiple individuals. While extensive research has focused on brain samples from cervid and transgenic mouse brains, less research has been dedicated to studying and characterizing peripheral prions, particularly from natural hosts, leaving a critical knowledge gap that this work addresses.

For this study, we assessed biochemical strain differences between paired obex and lymph node samples from naturally exposed white-tailed deer from Arkansas, USA. Here, we show that there are some significant differences between brain-derived and lymph node-derived prion isolates in some of our biochemical assays, but not all. Specifically, there were interesting electrophoretic differences between the brain and lymph-node samples and statistical differences in the glycoform ratio of PrP^{SC}. There were no differences between the brain and the lymph node in conformational stability. Surprisingly, we also found that there were more differences between individuals within the brain than in the lymph node. Together, this work suggests that there are different structural characteristics of lymph node and brain derived CWD prions. These results are important first examination of the differences in biochemical characteristics between brain and lymph node tissue and will inform future studies poised to assess strain differences using bioassay and other traditional prion assays.

Materials and Methods

Sample homogenization

Lymph node and obex samples from white-tailed deer that tested positive for chronic wasting disease (CWD) were provided frozen from the Arkansas Game and Fish Commission. Samples were stored at -20°C until processing. To ensure samples were not cross-contaminated, samples were trimmed with disposable scapple blades that were discarded after each sample. Samples were cut on a half of a petri dish that was also discarded after each sample was trimmed. Gloves and lab bench paper were also changed between each sample. Lymph node samples were then placed in homogenizing tubes with 7-10

zinc zirconium homogenizing beads and homogenized to 20% w/v in PMCA I buffer (1x PBS 150 mM NaCl, 4 mM EDTA) with cOmplete protease inhibitor (Roche). Samples were homogenized on a BeadBlaster for 10 rounds, with each round consisting of 3 cycles of a 30 sec pulse at 6 m/s followed by a 10 sec rest between each pulse. Samples were rested on ice for 5 min between each of the 10 rounds. Once samples were homogenized, samples were aliquoted and stored at -20°C until further use. Obex samples were also processed to 20% w/v homogenate in PMCA I buffer and protease inhibitor as described above, but obex samples were homogenized with 7-10 glass beads and for 2-3 rounds with a 5 min rest on ice between each round on the BeadBlaster. Samples were then aliquoted and stored at -20°C until use.

Conformational Stability Assay and Glycoform Ratio

To assess the conformational stability of the prions in the brain and the lymph node, samples were thawed and 15 µl of sample was added to 15 µl of GndHCl in 0.5 M increments from 0-4 M, briefly vortexed and incubated at room temperature for 1 hour. After the 1 h denaturation, samples were precipitated in ice-cold methanol overnight at -20°C. The following day, samples were removed from the -20°C, centrifuged at 13,000 rcf for 30 min at 4°C. Then, GndHCl and methanol were removed from the protein pellet and sample was resuspended in either 18 µl of PMCA I buffer (lymph node samples) or 36 µl of PMCA conversion buffer (1x PBS 150 mM NaCl, 4 mM EDTA, 1% Triton-X 100, obex samples). Lymph node samples then had 2 µl of 500 µg/mL of proteinase K (PK, Roche) (diluted in 1x PBS and 0.5 M EDTA) added for a final concentration of PK equal to 50 µg/mL in the sample. Obex samples had 4 µl of 1000 µg/mL of proteinase K (PK, Roche) (diluted in 1x PBS and 0.5 M EDTA) added for a final concentration of PK equal to 100 µg/mL PK. Samples were then incubated on a shaking heat block for 30 min @ 37°C and 800 rpm. Then samples were denatured in the presence of 3x loading buffer (200 µl 4x sample loading buffer [Invitrogen] and 80 µl of 10x sample reducing agent [Invetrogen]) for 10 min at 95°C. Samples were then either saved at -20°C or immediately run by western blot and analyzed for conformational stability and glycoform ratio.

Western blotting

Samples were run on 12% bis-tris gels [NuPage] in 1x MOPS running buffer and transferred to polyvinylidene difluoride membranes. Non-specific binding was reduced by blocking the membranes in

5% nonfat dry milk and 1% tween-20 in 1x PBS (NFDM) for 1 hour with rocking at room temperature. Membranes were then incubated in HRP-conjugated anti-PrP monoclonal antibody Bar224 (Cayman Chemical) diluted to 1:20,000 in SuperBlock (Thermo Fischer) overnight at 4°C. Blots were washed the following day in PBST (0.2% Tween 20 in 1x PBS) six times for 5 minutes each wash. Membranes were developed using enhanced chemiluminescent substrate (Millipore) for 5 minutes before imaging on ImageQuant LAS 4000 (GE).

Data analysis

Densitometric analyses were completed in ImageJ. Statistical analysis and graphing were performed in GraphPad Prism (version 8.30). Conformational stability was determined by calculating the concentration at which the signal was half of the input ($[GndHCl]_{1/2}$) after fitting the data to a fourth order polynomial regression. Glycoform ratio was calculated in ImageJ by determining what percentage of the total signal was contributed by each glycosylation state. Glycoform ratio data was arcsine transformed before analysis so percent data would fit a normal distribution and compared. Comparisons were made only between equivalent band types. For example, the diglycosylated band of one sample was only compared to the diglycosylated band from the other isolates. Different bands (e.g., di- and monoglycosylated) were not compared. Only samples that had at least three successful replicates (conformational stability) or had results replicated on at least two blots with three samples each (glycoform ratio) were included for analysis.

Results

Sample Origin and Result Overview

Samples used in this study were all collected from naturally exposed white-tailed deer in the state of Arkansas and shared with us from our collaborators from the Arkansas Fish and Game Commission. Of the nine animals that had paired obex and lymph node samples, only four of the animals gave us interpretable data from both the tissues that enabled us to compare intrahost variation (Table 2.1).

Table 2.1. Animal identification number and summary of biochemical strain typing results.

SAMPLE	OBEX		LYMPH NODE	
	CSA	*Glycoform Ratio*	*CSA*	*Glycoform Ratio*
10023	Yes	Yes	Yes	Yes
10074	Yes	Yes	Yes	Yes
10083	Yes	Yes	Yes	Yes
07399	Yes	Yes	Yes	Yes
07416	No	No	Yes	Yes
10030	No	No	Yes	Yes
14707	Yes	Yes	No*	No*
10080	No	No	No	No
07415	No	No	No	No

*Only 1 western blot of these samples (of 5 attempts) gave interpretable data. Too few replicates for conformational stability analysis or glycoform ratio to be determined. Samples were excluded from analysis. CSA = conformational stability assay.

Electrophoretic differences between obex and lymph node samples at ≥ 2.5 M GndHCl

Samples were prepped for analysis by conformational stability and glycoform ratio as described in the methods section. Samples were then run on a Western blot to collect densitometric and electrophoretic mobility data. Differences in electrophoretic mobility of a prion sample reveals structural differences that dictate PK accessibility, resulting in different PK-resistant core fragments of PrP^{Sc}. These structural differences are reliable biochemical indicators of different prion strains[19-21]. Samples were incubated in 0-4 M GndHCl in 0.5 M increments as described in the methods section. Obex samples that were incubated in 2.5 M GndHCl and greater migrated faster than samples that were exposed to lower concentrations of GndHCl (Figure 2.1). This was only observed in obex samples and was never observed in the lymph node samples and these data were consistent among all four individuals (Figure 2.1).

Conformational stability comparison between paired obex and lymph node samples

Different prion strains can be more or less conformationally stable in the presence of chaotropic denaturing agents like GndHCl. We compared the conformational stability of prions derived from lymph node or brain samples to determine if this strain characteristic would reveal strain differences from different tissues within the same animal. Samples were treated with GndHCl in increasing concentrations and fourth order polynomial regression was utilized to determine $[GndHCl]_{1/2}$ values. While one animal

trended toward statistical significance, there were no statistical differences between paired obex-derived and lymph-node derived prions in any of the four individuals examined (unpaired t-test, $p<0.05$, Figure 2.2). There was, however, a statistical difference in the variance between the obex and lymph node-derived prion samples in animal 10083 (F-test, $p<0.05$). Animal 10074 did not have significant differences in variance of the sample, but was trending towards significance (F-test, $p=0.07$). The other two samples did not have significant difference in sample variance (F-test, $p>0.05$).

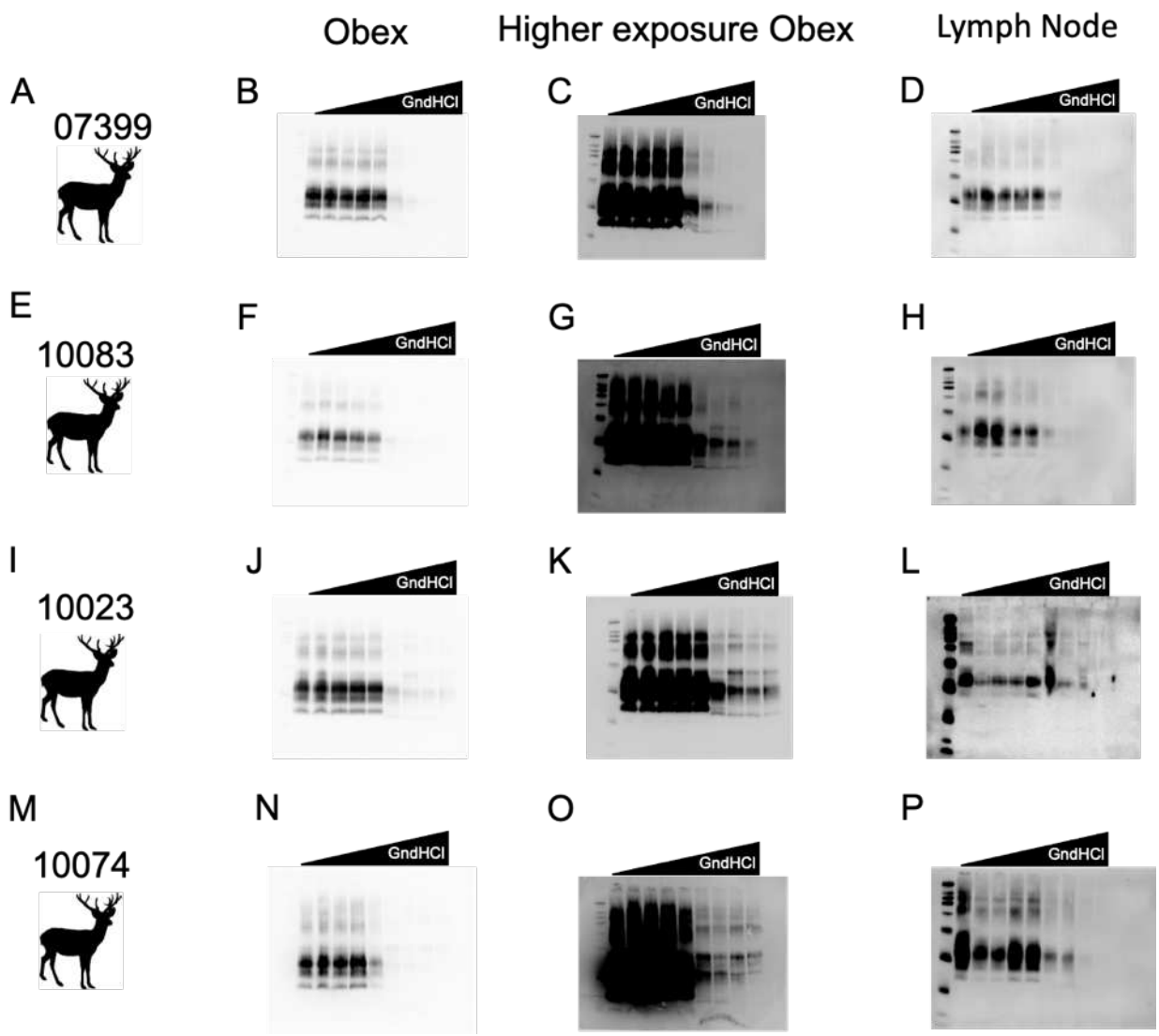

Figure 2.1. Electrophoretic differences detected between the obex and the lymph node of the same animal. Animal identification numbers for the white-tailed deer that were analyzed (A, E, I, M). Samples to the right of the animal identification are all from the same individual. Sample obex blots are shown at a typical exposure (B, F, J, N) and overexposed (C, G, K, O) show the unique electrophoretic signature noted in the obex sample at ≤ 2.5 M GndHCl. Representative conformational stability blots for lymph node samples are shown (D, H, L, P) and do not show the electrophoretic change at increased concentrations of GndHCl.

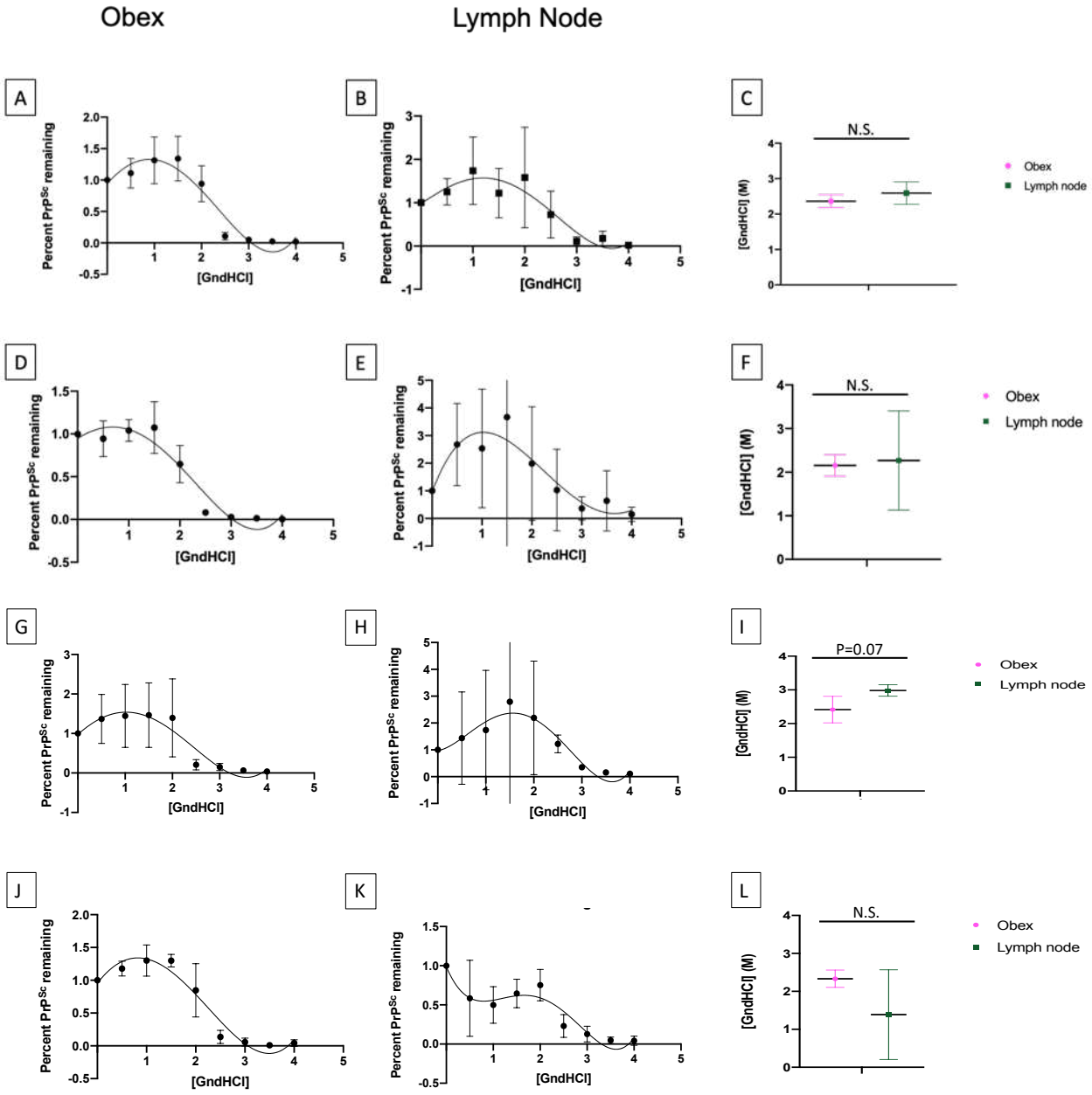

Figure 2.2. No differences in conformational stability of between obex- and lymph node-derived prions. No differences were observed between obex and lymph node samples from animals 07399 (A, B, C), 10083 (D, E, F), 10023 (G, H, I) or 10074 (J, K, L). Samples were treated with GndHCl as described in the methods section. Fourth-order polynomial regression curves are shown for both obex (A, D, G, J) and lymph node (B, E, H, K) samples and depict the average curve from at least 3 experiments. The curves show the mean and standard deviation of the samples at each concentration of GndHCl. Different y-axes are shown so the data is easily visualized and can more accurately depict the range of the standard deviation for the samples at each concentration of GndHCl. The mean and standard deviation of the $GndHCl_{1/2}$ values from the individual replicates for both the obex and the lymph node of the same animal are graphed (C, F, I L). While no statistical differences were found between sample means, the difference between obex and lymph node of sample 10023 is trending towards significance ($p=0.07$). Unpaired t-test, $p<0.05$.

Glycoform ratio differences between paired brain and lymph nodes

Next, glycoform ratios of obex and lymph node tissue samples were assessed. Significant differences were found between paired brain and lymph node samples in at least two glycosylation states for all four individuals examined (unpaired t-test, $p<0.05$, Figure 2.3).

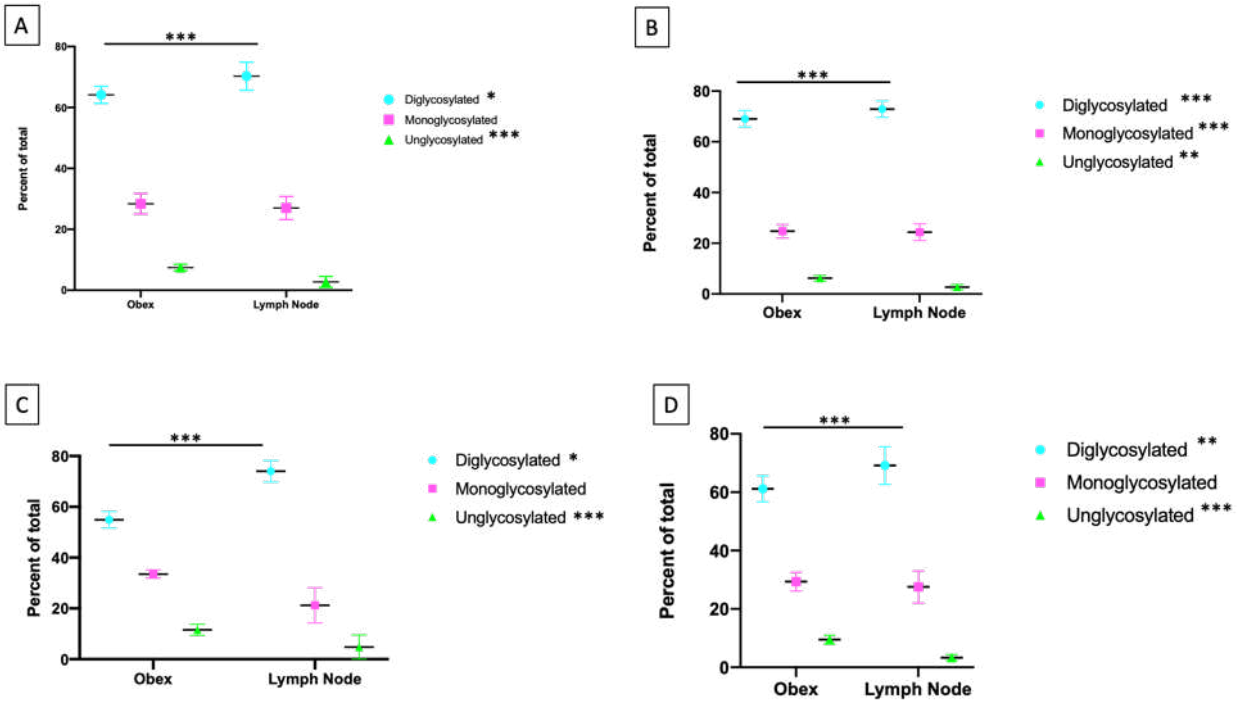

Figure 2.3. Significant differences in glycoform ratio between obex and lymph node samples of the same animal. Significant differences were found between obex and lymph node tissue in samples 07339 (A), 10023 (B), 10083 (C), and 10074 (D). Mean and standard deviation are show. Asterisks by the legend indicate if there were significant differences in the di-, mono- and/or unglycosylated band and indicate the level of significance. Unpaired t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Conformational stability and glycoform ratio comparison between brain and lymph node

To determine if significant biochemical differences exist between brain and lymph node prions generally across individuals, we compared mean values for combined conformational stability and glycoform ratio data for each tissue from all individuals. We observed no significant differences when comparing $[GndHCl]_{1/2}$ values from brain and lymph node (paired t-test, $p<0.05$; Figure 2.4). While we observed no statistical differences between mean $[GndHCl]_{1/2}$ values between brain and the lymph node samples, lymph node samples exhibited statistically more variance in mean $[GndHCl]_{1/2}$ than obex samples (F-test, $p<0.05$). When comparing glycoform ratio between obex-derived and lymph node-derived prions, there were statistical differences in glycoform ratio across all three glycosylation states (paired t-test, $p<0.001$; Figure 1.5).

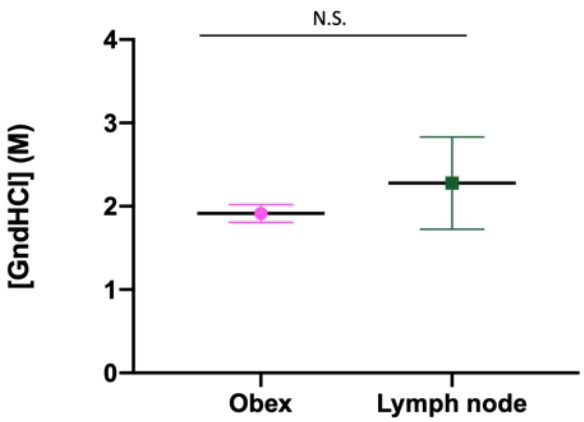

Figure 2.4. No difference in conformational stability between obex and lymph node samples. To see if there were overall tissue differences between the obex and lymph node-derived prions, samples that had interpretable data for both tissues were compared and no differences were found between the mean (paired t-test, $p>0.05$), but there were significant differences in the variance (F test, $p<0.05$). Graph is showing the mean and standard deviation of the obex and lymph node samples. Obex, n=5; lymph node, n=6.

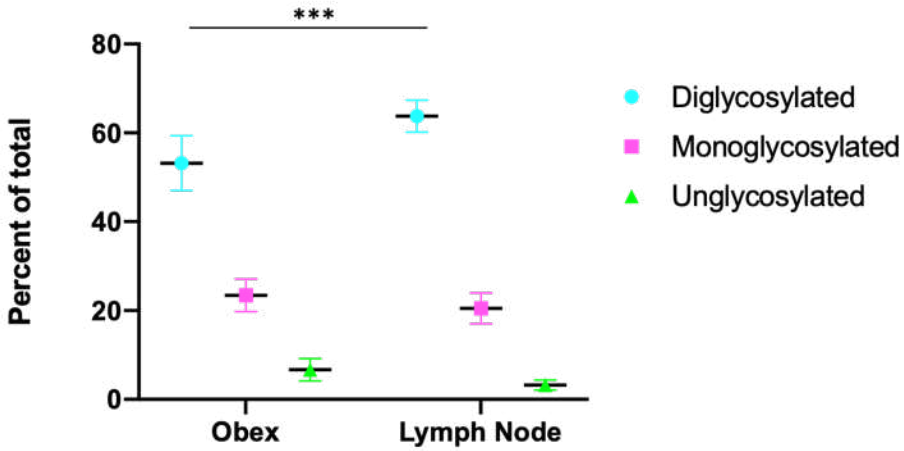

Figure 2.5. Significant differences in PrP^{SC} glycofrom ratio between obex and lymph node-derived prions. Significant differences between the di-, mono- and unglycosylated bands were all highly significant (paired t-test, $p<0.001$). Mean and standard deviation are shown. Obex, n=5; lymph node, n=6.

Tissue-specific conformational stability and glycoform ratio comparison between individuals

To assess if there were differences in conformational stability and/or glycoform ratio between individuals in either the brain or the lymph node, isolates originating from the same tissue were compared across individuals. $[GndHCl]_{1/2}$ values were compared between all animals that had interpretable data, not only the four samples that allowed for within animal comparison (Table 2.1). There were no statistical differences in conformational stability ($[GndHCl]_{1/2}$) in prions derived from either brain or lymph node when compared among individuals (Figure 2.6). There were few differences in glycoform ratio in lymph node samples when compared between individuals (ANOVA with Tukey adjustment; Figure 2.7, Table 2.2.). However, many glycoform ratio differences were detected among individuals from obex-derived prions. In fact, there were only two samples that, when compared to each other, were not statistically different (ANOVA with Tukey adjustment; Figure 2.7, Figure 2.8, Table 2.3.).

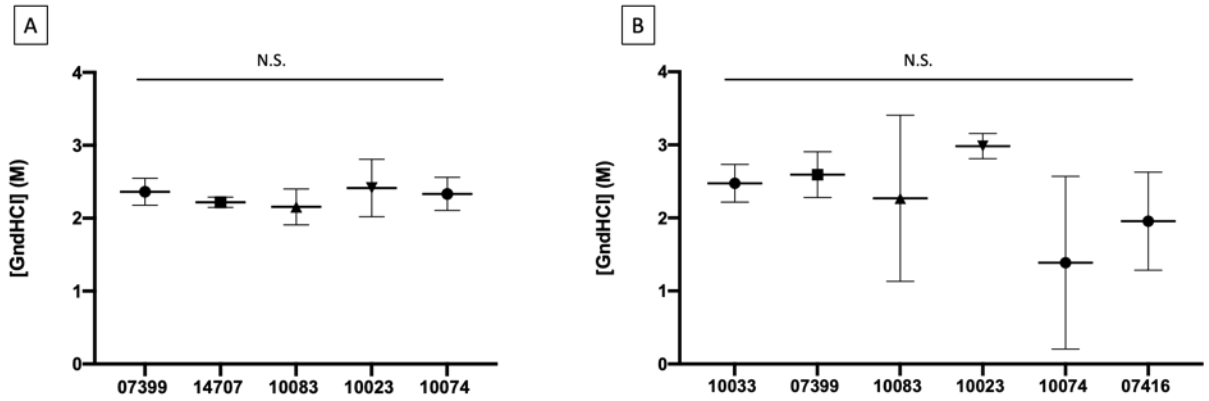

Figure 2.6. No conformational stability differences between individuals in either obex or lymph node derived samples. There are no differences between obex samples between any individuals (A). There are no differences between lymph node samples between individuals (B). One-way ANOVA with Tukey adjustment. N.S. = not significant ($p>0.05$).

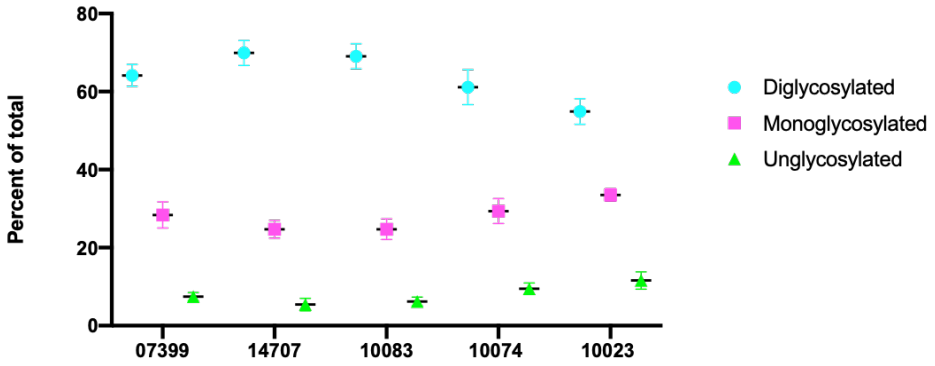

Figure 2.7. Significant differences in individual obex sample glycoform ratios. Samples are shown individually. A summary of the multiple comparisons and significant differences between samples are shown and summarized in Table 2.3.

Table 2.2. Multiple comparisons between individual obex samples.

	Diglycosylated		Monoglycosylated		Unglycosylated	
Sample comparison^	p value	Significance	p value	Significance	p value	Significance
07399 vs. 14707	0.0066	**	0.0456	*	0.0176	*
07399 vs. 10083	0.0173	*	0.0251	*	0.3450	ns
07399 vs. 10074	0.3187	ns	0.9244	ns	0.0678	ns
07399 vs. 10023	<0.0001	****	0.0011	***	<0.0001	****
14707 vs. 10083	0.9968	ns	0.9993	ns	0.6447	ns
14707 vs. 10074	<0.0001	****	0.0051	**	<0.0001	****
14707 vs. 10023	<0.0001	****	<0.0001	****	<0.0001	****
10083 vs. 10074	<0.0001	****	0.0025	**	0.0003	***
10083 vs. 10023	<0.0001	****	<0.0001	****	<0.0001	****
10074 vs. 10023	0.0022	**	0.0119	*	0.1001	ns

^One-way ANOVA with Tukey adjustment.

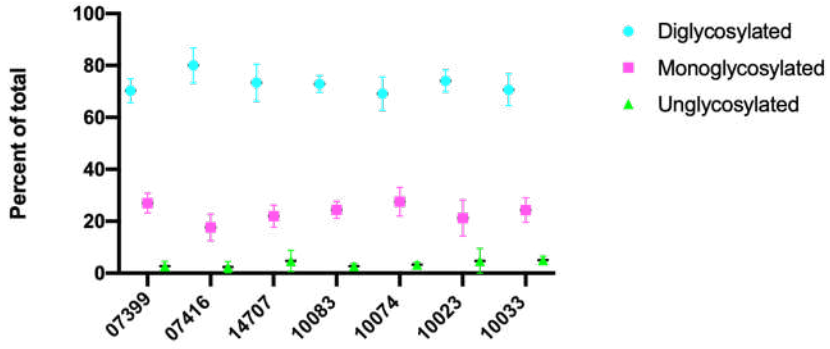

Figure 2.8. Few differences in individual lymph node sample glycoform ratios. Samples are shown individually. A summary of the multiple comparisons and significant differences between samples are shown and summarized in Table 2.3.

Table 2.3. Multiple comparisons between individual lymph node samples.

	Diglycosylated		Monoglycosylated		Unglycosylated	
Sample comparison^	p value	Significance	p value	Significance	p value	Significance
07399 vs. 10083	0.9514	ns	0.9369	ns	0.9996	ns
07399 vs. 10023	0.7472	ns	0.1899	ns	0.5652	ns
07399 vs. 07416	0.0124	*	0.0107	*	0.9990	ns
07399 vs. 10033	>0.9999	ns	0.9204	ns	0.2456	ns
07399 vs. 10074	0.9979	ns	>0.9999	ns	0.8841	ns
10083 vs. 10023	0.9991	ns	0.8572	ns	0.8754	ns
10083 vs. 07416	0.2354	ns	0.2354	ns	0.9926	ns
10083 vs. 10033	0.9900	ns	>0.9999	ns	0.5846	ns
10083 vs. 10074	0.8418	ns	0.8949	ns	0.9932	ns
10023 vs. 07416	0.3617	ns	0.8279	ns	0.5411	ns
10023 vs. 10033	0.9168	ns	0.8781	ns	0.9907	ns
10023 vs. 10074	0.5528	ns	0.1598	ns	0.9798	ns
07416 vs. 10033	0.0687	ns	0.2556	ns	0.2635	ns
07416 vs. 10074	0.0065	*	0.0092	**	0.8289	ns
10033 vs. 10074	0.9964	ns	0.8735	ns	0.7688	ns

^One-way ANOVA with Tukey adjustment.

Discussion

Chronic wasting disease is an invariably fatal disease infecting cervids world-wide. This disease is devastating to the individual animals that become infected and has resulted in substantial population-level effects in free-ranging animals, including population decline and herd culling as a method of CWD control[39–44]. The unique nature of prions and prion diseases, coupled with extremely facile animal-to-animal transmission, necessitates a thorough understanding of the pathogen causing disease.

Because CWD and other prion disease are neurological, with the highest prion titers occurring the nervous tissue, most prion research has focused on brain-derived samples. While these pivotal studies are critical to our current understanding of disease, the profound lymphotropism and presence of infectious prions in extraneural sites should be considered when investigating CWD deposition into the environment, cervid transmission and potential zoonotic transmission. Furthermore, most studies that have focused on prion strain characterization have utilized mouse bioassay, where prion isolates are passaged into transgenic mice expressing PrP^C from another species. The resulting disease phenotype in the mouse and the biochemical characteristics of the prions from the brains of infected mice are assessed

to give a set of disease characteristics that are then defined as a prion strain[19,20]. These studies are essential and critical to our understanding of prion diseases; however, the resulting strain that stabilizes and emerges may be quite different from the traits of the input isolate. Few researchers have thoroughly investigated prion strain characteristics, especially, before passaging the sample into a model organism. Moreover, it is unclear how other host factors influence the outcoming strain[45] and the logistical and financial constraints make widespread strain characterization challenging. One of the relevant and important pieces of this work is to look at the infectious prion from the natural host before passing into a mouse. These experiments looking at the infectious agent from the natural host are critical to our understanding of the biochemical differences between lymph node and brain-derived prions. Any differences that we find have important implications for CWD transmission and disease progression.

Of the paired samples that were received for biochemical strain typing analysis, all the data shown here were from animals that were confirmed CWD positive before samples were sent to us. Of the nine samples that were received, only four samples had interpretable data for both obex and lymph node tissues for intra-animal comparison. Work optimizing our assays to get the clearest, most reproducible data, indicated that brain samples needed to be PK digested in the presence of a detergent (in these experiments, 1% Triton-X 100), with a higher concentration of proteinase K (PK) (100 µg/mL), and a lower amount of protein (5% w/v homogenate) needed to be run on the gel (data not shown). Lymph node samples ran well when more protein was loaded onto the get (10% w/v homogenate) and digested in less PK than the obex samples (50 µg/mL). If brain/obex samples were processed as the lymph nodes were, the prion signal was indistinguishable from PrP^{C}, thus different digestion conditions were employed. This was necessary to get comparable data, but it is also another important note about the strain differences observed between the obex and lymph node samples. It is well documented that there is more PrP^{C} in the brain than in other peripheral tissues and that highest PrP^{C} expression levels are found in the brain and lymphoid tissues[46–48]. It could be that there is too much PrP in the brain to give accurate results when not more intensely digested. Additionally, the PrP^{Sc} in the brain may form denser plaques that are at too high of a concentration in a sample are unable to be digested by PK without the extra dilution, detergent and amount of PK. Future directions to determine if the structures are different could involve more extensive

epitope mapping and/or electron microscopy. This difference between obex and lymph node samples was the first biochemical difference that was noted.

It is interesting and important to consider the samples that had incomplete data for this analysis. Samples 07416 and 10030 had interpretable data for only PrP^{Sc} derived from lymph node samples, but not obex samples. CWD replicates to detectable levels in lymph nodes before it is detected in the brains[14,18,49]. For this reason, it is possible that there are simply not enough prions in the sample yet to be detected by our relatively low-sensitive Western blot assay. It is also possible that the prions that are in the brain are still protein oligomers rather than PK-resistant plaques that are undetectable by our assays as well. Additional work needs to be done to determine the prion titer in these animals, if there is infectious material present, and what the phenotype of disease is upon passage into animals.

In addition to the animals that had positivity in the lymph node but not the brain, there was one animal, 14707, that only gave us positivity in the brain and not in the lymph node. Typical prion signal from this sample was detected in one replicate, thus we can presume that the prions in this tissue are similar to the other samples that were examined here; however, because the data were not repeatable, they were excluded from the analysis. It would be very surprising to have an animal that had typical PK-resistant prion positivity in the brain but not in the lymph node. It is also possible that difficult nature of working with lymph node samples precluded the gathering of the data. Difficulty with tissue homogenization and western blotting were not insignificant and are likely contributing factors to the difficulties of working with that sample. Finally, there were two samples that did not give interpretable data for either the brain or the lymph node. These samples likely had prion levels that were below the limit of detection by western blot and were not able to be analyzed in this study.

For the four samples that did have interpretable, reproducible data in both tissues, there were differences that were noted in the electrophoretic pattern of the obex samples and the lymph node samples starting at 2.5 M GndHCl. Notably, for all of the obex samples analyzed, 2.5 M and higher samples shift farther down the gel, suggesting that the prions are being denatured in a unique way and allowing proteinase K to have differential access to the protein before it is entirely digested. This electrophoretic change was not observed in the lymph node samples (Figure 2.1). These results suggest that there may be a structural difference between the prions from the brain and the lymph node that is

revealed when treated with ≥2.5 M GndHCl. Alternatively, the difficulties of working with lymph node samples may have precluded us from having a clean enough blot to see this shift. This is not likely the case as other prion isolates in the lab that are well characterized (E2) do not show this signature and, in fact, resembles PrP^C at high concentrations of GndHCl (Figure A.1). Whether this biochemical difference translates to a biological significance remains to be determined, but it is an interesting line of evidence that there is something structurally different between obex- and lymph node-derived prions. Utilizing different antibodies that bind to unique epitopes on western blot can also be used to see structural difference for alternative PK digestion site usage, which is a likely explanation for the electrophoretic change seen in Figure 2.1. There are also a lot of high-molecular weight proteins staining for PrP, which may represent prion oligomers, but this was consistent between both obex and lymph node samples (Figure 2.1).

Next, we compared the conformational stability between the obex and lymph node prion samples. There were no significant differences in the mean $[GndHCl]_{1/2}$ values between the obex- and lymph node-derived prion samples (Figure 2.2). Because the samples studied in this project are from a natural host and more challenging to work with than, for example, transgenic mouse brains or cell culture-based assays, we are perhaps unable to detect subtle differences between brain and lymph node strains. CWD is traditionally one of the more stable prion strains and it may be that a characteristic of CWD overall is that they are very stable in the presence of GndHCl. These data do indicate that CWD prions, regardless of the tissue of origin, are stable in the presence of denaturing agents.

While there was no difference between the average $[GndHCl]_{1/2}$, there was more variability in the lymph node-derived prion samples as evidenced by the large error bars at each concentration of GndHCl (Figure 2.2). Moreover, there were there were unequal variances between the brain and lymph node $[GndHCl]_{1/2}$ of one animal (10083) and nearly a significant difference in another (10074), indicating that there was more variability in the lymph node-derived prions. Based on our hypothesis that there is greater strain diversity in the lymph node sample, the more variability that we see in the lymph node may be due to a larger and more diverse population of prion strains in this extraneural site. The difficulty of working with lymph node sample may have played a small role in the increased variability that we see in our extraneural samples, but this does imply that there is greater diversity of strains in the lymph node.

Finally, the denaturation curves look different between the brain and lymph node samples, potentially indicating that there are some biochemical differences between the two tissues that aren't entirely represented in the simple average (Figure 2.2). Future studies should be done to see if these biochemical differences have any biological significance.

Next, we analyzed paired samples by glycoform ratio. There are significant differences between the obex and lymph node-derived samples in all four animals that were tested (Figure 2.3). This is an important indicator of different prion strains and is another line of evidence that the prions present in the lymph node differ somewhat to those in the obex of the same animal. These differences could signify a biochemical strain difference between these animals; however, there is no systematic assessment of the glycosylation pattern of PrP^C in the lymph node or brain of white-tailed deer. So, while these data are indicative of a biochemical strain difference, we cannot, at present, rule out that the populations of glycosylated PrP^C are very different in the lymph node and brain and could be influencing the data. Even if this is the case, it is likely still an important and relevant aspect to prion propagation in the periphery and may be a biochemical difference with biological ramifications. In fact, a recent paper from the Baskakov lab has proposed the differential glycosylation patters are drivers of prion strains[50]. Assessment of glycosylation differences in PrP^C could be analyzed using 2D gels. Future studies with *in vivo* models or observational studies of infected deer in the field will be necessary to determine if this biochemical difference is biologically relevant.

Next, individual animals were averaged together to see if there were any tissue differences between the obex and lymph node samples. There were no statistical differences in conformational stability, but there was a significant difference in the variance between the samples (Figure 2.4). There were also significant differences in glycoform ratio when brain and lymph node samples were compared (Figure 2.5). These results mirrored the results that were seen on an individual level and again suggest that there are biochemical strain differences between obex and lymph node CWD prions.

Lastly, biochemical strain differences were assessed between individuals in the same tissue to identify any differences between animals. There were no differences in conformational stability between any of the obex samples or lymph node samples (Figure 2.6). These data suggest that while there are some indications of strain differences between tissues, there do not appear to be any strain differences

between individuals. When individual animals are compared across individuals by tissue type, there are few differences in glycoform ratio in the lymph node samples (Figure 2.8, Table 2.2). All of the significant differences are between sample 07416 and another sample. This result may indicate that there is something slightly different in glycoform ratio in that sample, but the results for that sample were not as clean and easy to interpret as some of the other samples (data not shown). This could be contributing to the observed differences. Surprisingly, there were many differences between individuals when obex glycoform ratios were compared (Figure 2.7, Table 2.3). This data suggests that different prion strains were selected in the lymphoid organs and trafficked to the brain where a more homogenous “cloud” of prion strains was present and a more uniform population of prions was propagated. This group of prions is more homogeneous and perhaps allows for differences between individuals.

Taken together, the data presented here provide some evidence for biochemical strain differences between obex and lymph node derived prions. While this data does support that there are some biochemical differences between these two tissues, future directions that include mouse bioassay are required to see if these biochemical differences translate to a biological difference. This work has important implications to our understanding within host strain differences. These data suggest that we should spend more time characterizing and studying extraneural prions and prion strains as they differ from neurogenic prions and are more likely to be the prions that are being shed into the environment and exposing other cervids and humans to prions.

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Chapter 3:

Characterization of chronic wasting disease isolates from natural hosts demonstrates strain diversity and identifies potential novel strains

Summary

Chronic wasting disease is a relatively new prion disease, only having been observed for the first time in captive mule deer populations in the late 1960s. Since the initial publication, CWD has exploded worldwide, most recently expanding to infect cervids in Europe in 2016. The effects of CWD on cervid populations are devastating and some mathematical models even predict that uncontrolled CWD spread could result in population extinction events. While the origin of CWD is unknown, there are two well-regarded hypotheses: 1) that scrapie from sheep was able to cross the species barrier and transmit to mule deer and 2) that CWD arose through a spontaneous conversion event and was then subsequently transmitted from animal to animal. Furthermore, it was often thought that all cases of CWD were the result of CWD being transported from the initial outbreak. Indeed, this is the case in a number of outbreaks, but there are many locations that now have endemic CWD with no know or clear connection to Colorado. This has raised the question as to whether there are multiple strains of CWD that are causing these outbreaks. A lot of excellent research has been done on chronic wasting disease strains, but all of this work has come from using primary isolates passaged into mouse models, which may be influencing the resulting prion. Furthermore, these studies often do not include numerous sources of inocula, limiting the scope of our understanding of the strain diversity. There has also been no systematic assessment of CWD isolates from multiple outbreak foci. To best control CWD, it is imperative that researchers, wildlife management agencies and community stakeholders have a thorough understanding of the infectious agent causing the disease such that the most informed management practices can be put into place. The purpose of this study was to use biochemical prion strain typing techniques to determine if there are different prion strains circulating from multiple epizootics. We found a number of interesting isolates from different locations, as well as some differences between locations and between captive and free ranging animals, implicating novel strain circulation and evolution. Overall, this work highlights the diversity of

CWD prion strains circulating in North America and emphasizes the importance of continuing research to understand the etiologic agent of chronic wasting disease.

Introduction

Chronic wasting disease (CWD) is a devastating prion disease that was first discovered in the late 1960's and first described in the literature in the 1980's[1,2]. Since that time, CWD has spread worldwide, infecting both captive and free ranging cervids[1,3–5]. A common assumption has been that all cases of CWD have originated from the initial cases of CWD, implying that all cases of CWD are of the same etiological agent and CWD is a homogeneous disease radiating from the initial outbreak. While transplanted animals have been known to be the source of some CWD cases/outbreaks, such as in South Korea where transport of CWD-infected elk was known to be the origin of CWD in their captive populations[3], there are other CWD outbreaks that are of unknown origin (such as those in Scandinavia[4–6]) that have now been shown to be distinct prion strain[5]. Because CWD outbreaks have been documented that have no connection back to Colorado or other infected animal transport, it has raised the question as to whether CWD is as homogeneous of a disease as originally believed or if there are multiple strains of CWD circulating in different CWD outbreaks. Chronic wasting disease is a relatively new disease, being observed and described only within the last 60 years. While other prion diseases have been observed for much longer, such as sheep scrapie, it is possible that this prion disease is still evolving and changing and multiple prion strains may be emerging. While prion strains are generally epigenetic, differences in *PRNP* genotype can contribute to strain differences and strain emergence in some cases[7], but different strains can also emerge without a change in genotype.

Defining a prion strain is a substantial challenge in the field because it is difficult and not well agreed upon how different isolates need to be to qualify as separate strains. In part, defining strains is difficult because prions are able to epigenetically transmit unique disease phenotypes without necessitating a difference in the *PRNP* gene. Genetic changes are often utilized in defining differences in more traditional pathogens, but a different set of techniques is required to assess prion strains. Indeed, an excellent definition of a prion strain was defined in Bartz (2016), where he defined a prion strain as "phenotype of disease under a fixed set of host and agent parameters"[8]. To characterize a phenotype of disease, however, it requires the prion isolate to be transmitted to a model organism, often a transgenic

mouse model, and serially passaged to achieve strain stabilization. Then, prion strains are characterized for biological features (including days to terminal disease, lesion profiling in the brain, and disease presentation), as well as biochemical characteristics (including glycosylation pattern, conformational stability, PK resistance and electrophoretic mobility)[8–10]. While transgenic mice and biological characterization of prions have been exceptionally important in advancing the understanding of prion diseases and characterizing prion strains, the paucity of data on prion strains from a natural host is a significant knowledge gap in the field. Furthermore, the ability to detect prion strain differences without need for bioassay would enable more affordable and less time-consuming methods for agencies to characterize circulating CWD strains.

Multiple strains of CWD have been characterized from North American CWD isolates. One study noted that there are two distinct CWD strains, denoted as CWD-1 and CWD-2, where CWD-2 had unilateral PrP^{Sc} deposition and longer incubation times[11]. Additionally, Wisc-1 is a strain of CWD that described and characterized, but the authors of the paper suspect that Wisc-1 is very similar to CWD-1 and any differences observed were the result of using a different transgenic mouse line to characterize the prion strain. Because of the confounding issues of using different transgenic mice, it would be difficult to ascertain if they are indeed the same prion strain because of the use of a different model[12]. For the purposes of this research, isolates examined here will be compared to “classical” CWD based upon the biochemical findings from Angers *et al.*[11] and Velasquez *et al.*[12]. Biochemically, classical CWD is primarily diglycosylated and is quite stable in the presence of chaotropic denaturing agents (~2-2.5 M $[GndHCl]_{1/2}$).

Distinct from classical CWD, H95+ has also been described as a unique prion strain that emerged when white-tailed deer (H95/96wt or H95/96S) infected with CWD were passaged into Tg60 mice (white-tailed deer PrP, 96S), but there was not a unique strain phenotype when passaged into Tg33 mice (mule deer PrP, 96GG). The H95+ CWD strain represents the emergence of a prion strain dependent on genotype and, interestingly, H95+ was transmissible to wild-type mice, suggesting that this strain may have more zoonotic potential than other CWD strains[12,13]. This research also highlighted how genotype could play a role in novel strain emergence. Interestingly, cervid populations that have had CWD are beginning to show selection for resistant genotypes[14,15]. The emergence of the H95+ strain

suggests that genetic drift towards resistant genotypes may lead to the emergence of novel, and perhaps more zoonotic, CWD strains in certain subpopulations[14].

While mouse and other animal models have been essential to prion strain typing, the lack of strain characterization from a naturally infected host prior to passage in a mouse model impairs the understanding of what is happening in the infected cervids. In fact, cofactors have been shown to be essential to the maintenance of prion strain properties and it is unclear if mice or other model organisms may be influencing the strain properties of CWD as it is passaged (and serially passaged)[16]. There is also research to suggest that host factors independent of genotype play a role in strain emergence, suggesting that model organisms could be influencing the emergence of CWD strains from these experiments[17].

Generally, classical, North American CWD has been found to be quite similar, perhaps in part a result of some of the downsides to relying so heavily on model organisms to characterize prion strains or because all outbreaks do stem from the initial cases from Colorado and little strain evolution has occurred over time. It is also possible that there is far more heterogeneity than previously thought, perhaps from multiple introduction events or cross-species transmission of scrapie to deer or spontaneous disease outbreaks that simply haven't been characterized. It is also possible that there is quite a bit of prion strain diversity within an animal that isn't observed in the brain that is being missed when strain typing prion diseases. CWD is a profoundly lymphotrophic prion disease and perhaps there are more quasi-species in these peripheral tissues that are being deposited into the environment and transmitted to additional animals[18–22] (i.e., the prion cloud hypothesis[23]). If true, cervids are exposed to numerous strains and it is possible that novel strains evolve regularly. Furthermore, mouse studies often focus on only a few sources of inocula (for animal number and cost constraints) to isolate and characterize prion strains[12,13,24]. It is possible that by using only a few animals to assess prion strains that a lot of strain heterogeneity has been missed. Any of these possibilities may be true, but there has never been a systematic assessment of CWD strain diversity prior to passage through a mouse model to examine this question. A better understanding of strain diversity could inform management strategies to mitigate CWD in a location (for example, if a prion strain is less stable than another, it is possible that the environment would hold infectious prions for less time, thereby reducing the amount of time that deer farms/environments would

need to be kept cervid-free). Knowledge like this can only be gained thorough analysis of multiple natural isolates from several locations, as done in this study.

Because of these knowledge gaps, this research project was started and aimed at characterizing CWD strain diversity from numerous CWD epizootics across the USA utilizing biochemical techniques. Isolates were obtained from Michigan, Missouri, Arkansas, Virginia, Iowa as well as captive animals from multiple deer farms across the US. Mostly lymph node isolates were obtained, but some brain samples were also submitted. These results suggest that there is some diversity of CWD prions when assessed by biochemical techniques both within and between locations, but that CWD is, generally, biochemically consistent with classical CWD. For this project, we were also fortunate enough to receive samples from many free-ranging animals, but also from several captive animals from deer farms across the US. We hypothesized that captive deer would have fewer biochemical differences and a more homogenous strain circulating within deer farms. We think this because deer farms are facilities in which the same strain of CWD may be circulated repeatedly over time and because there are fewer prion strains being introduced (from other conspecific cervids, for example) and a confined range in which the animals can move. To address this question, we compared samples from farmed deer to other free-ranging and did find some biochemical differences, supporting the hypothesis of evolution within deer farms. These results are important to provide state agencies and stakeholders with preliminary findings for them to continue to investigate CWD from locations with prion isolates with a unique biochemical signature. Future work assessing differences in biological outcomes of these biochemical differences will be essential to understand if these differences have disease implications, both for cervid populations and for potential transmission to human populations.

Materials and Methods

Sample homogenization

Retropharyngeal lymph node (LN) and brain (B) samples from white-tailed deer that tested positive for chronic wasting disease (CWD) were provided frozen from collaborators from Michigan (LN), Missouri (LN), Arkansas (LN and B), Virginia (LN), Iowa (LN), and multiple deer farms from the USDA (B). Samples were stored at -20°C until processing. To ensure samples were not cross-contaminated, samples were trimmed with disposable scapple blades in a half of a petri dish that were discarded after

each sample. Gloves and lab bench paper were also changed between each sample and only samples from a single location were processed on a single day. Lymph node samples were then placed in homogenizing tubes with 7-10 zinc zirconium homogenizing beads and homogenized to 20% w/v in PMCA I buffer (1x PBS 150 mM NaCl, 4 mM EDTA) with cOmplete protease inhibitor (Roche). Samples were homogenized on a BeadBlaster 24 (BenchMark Scientific) for 10-11 rounds, with each round consisting of 3 cycles of a 30 sec pulse at 6 m/s followed by a 10 sec rest between each pulse. Samples were rested on ice for 5 min between each of the 10 rounds. Once samples were homogenized, samples were aliquoted and stored at -20°C until further use. Brain samples were also processed to 20% w/v homogenate in PMCA I buffer and protease inhibitor as described above, but obex samples were homogenized with 7-10 glass beads and for 2-3 rounds with a 5 min rest on ice between each round. Samples were then aliquoted and stored at -20°C until use.

Glycoform ratio

Samples were obtained from the freezer, thawed and diluted to 10% w/v in either PMCA I (1x PBS 150 mM NaCl, 4 mM EDTA; lymph nodes) or PMCA II (1x PBS 150 mM NaCl, 4 mM EDTA, 1% Triton-x 100; brains) buffer. Samples were then digested in either 50 µg/mL (LN) or 100 µg/mL (B) in proteinase K (PK, Roche) and 0.05 M EDTA on a shaking heat block for 30 min @ 37°C and 800 rpm. Following PK digestion, samples were denatured in the presence of 3x loading buffer (200 µl 4x sample loading buffer [Invitrogen] and 80 µl of 10x sample reducing agent [Invitrogen]) for 10 min at 95°C. Samples were then either saved at -20°C or immediately run by western blot. Brain samples were not received until after lymph node samples and when trying to use the same methods to digest and analyze the samples, it was determined that the methods that worked for the lymph node samples were not sufficient for brain samples. For these reasons, the methods were optimized to obtain analyzable data for both tissues.

Lymph node samples analyzed for glycoform ratio occasionally had “pinchy” samples when run on a western blot and were difficult to analyze. This was likely the result of an excess of DNA in the samples. For this reason, samples that gave difficult or uninterpretable data were treated with DNase to improve the signal. Samples were incubated with 16 units of DNase (Sigma) on a shaking heat block for

1h at 37°C and 800 rpm. Samples were then digested in PK and denatured as above and run by western blot as described.

PNGase F digestion

When running samples by western blot, a doublet signal was noted in the diglycosylated band from some free-ranging white-tailed deer lymph nodes. To determine if the signal was the result of alternative PK digestion sites, samples were first PK digested in 50 µg/mL of PK for 30 min at 37°C and 800 rpm on a shaking heat block. Immediately after the PK digestion, PK was inactivated by heating the sample at 99°C for 10 min. Then, PNGase F digestion was performed following the manufacturer's instructions (New England Biolabs). Briefly, 9 µl of PK-digested lymph node or brain homogenate was added to 1 µl of 10x glycoprotein denaturing buffer and the sample was again heated at 99°C for 10 min and samples was then cooled to 37°C for 10 min. PNGase F master mix was prepared according to the manufacturer's instructions and 10 µl of master mix was added to 10 µl of the sample and incubated at 37°C and 500 rpm for 1h on a shaking heat block. Then, to prepare samples for western blot, 10 µl of 3x loading buffer was prepared as above, added to the sample and then samples were incubated at 95°C for 10 min. Samples were then run by western blot as described above.

Conformational Stability Assay

To assess the conformational stability CWD prions in either brain or lymph nodes, samples were thawed and 15 µl of sample was added to 15 µl of Guanidine hydrochloride (GndHCl) in 0.5 M increments from 0-4 M, briefly vortexed and incubated at room temperature for 1 hour. After the 1 h denaturation, samples were precipitated in ice-cold methanol overnight at -20°C. The following day, samples were removed from the -20°C, centrifuged at 13,000 rcf for 30 min at 4°C. Then, GndHCl and methanol were removed from the protein pellet and sample was resuspended in either 18 µl of PMCA I buffer (lymph node samples) or 36 µl of PMCA conversion buffer (1x PBS 150 mM NaCl, 4 mM EDTA, 1% Triton-x 100, obex samples). Lymph node samples then had 2 µl of 500 µg/mL of proteinase K (PK, Roche) (diluted in 1x PBS and 0.5 M EDTA) added for a final concentration of PK equal to 50 µg/mL in the sample. Obex samples had 4 µl of 1000 µg/mL of proteinase K (PK, Roche) (diluted in 1x PBS and 0.5 M EDTA) added for a final concentration of PK equal to 100 µg/mL PK. Samples were incubated on a shaking heat block for 30 min @ 37°C and 800 rpm. After the PK digestion, samples were denatured in the presence of 3x

loading buffer (200 µl 4x sample loading buffer [Invitrogen] and 80 µl of 10x sample reducing agent [Invitrogen]) for 10 min at 95°C. Samples were then either saved at -20°C or immediately run by slot blot or western blot and analyzed for conformational stability. Glycoform ratio data was also collected from some samples that were run by Western blot and gave interpretable data for glycoform ratio analysis.

Western blotting

PK-digested and denatured samples were run on 12% bis-tris gels [NuPage] in 1x MOPS running buffer and transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was reduced by blocking the membranes in 5% nonfat dry milk and 1% tween-20 in 1x PBS (NFDM) for 1 hour with rocking at room temperature. Membranes were then incubated in HRP-conjugated anti-PrP monoclonal antibody Bar224 (Cayman Chemical) diluted to 1:20,000 in SuperBlock (Thermo Fischer) overnight at 4°C. Blots were washed the following day in PBST (0.2% Tween20 in 1x PBS) six times for 5 minutes each wash. Membranes were developed using enhanced chemiluminescent substrate (Millipore) for 5 minutes before imaging on ImageQuant LAS 4000 (GE).

Slot blot

Samples that were processed for conformational stability assay were run and analyzed using a slot blot. Samples were loaded into the slot blot loaded with an activated PVDF membrane and vacuumed until the sample liquid was dried onto the membrane. Samples were briefly washed in PBST (0.2% Tween20 in 1x PBS) before being briefly reactivated in methanol. Samples were again washed 1x in PBST before being blocked in 5% nonfat dry milk and 1% tween-20 in 1x PBS (NFDM) for 1 hour with rocking at room temperature. Membranes were then incubated in HRP-conjugated anti-PrP monoclonal antibody Bar224 (Cayman Chemical) diluted to 1:20,000 in SuperBlock (Thermo Fischer) overnight at 4°C. Blots were washed the following day in PBST six times for 5 minutes each wash. Membranes were developed using enhanced chemiluminescent substrate (Millipore) for 5 minutes before imaging on ImageQuant LAS 4000 (GE).

Genotyping

Isolates from Missouri were genotyped to determine sequence and determine the codon at position 96 of the *PRNP* gene. Lymph node isolates were trimmed and DNA extracted using a DNeasy kit following manufacturers protocols (Qiagen). Samples were then PCR amplified using the primers 223F

and 224R from O'Rourke *et al.* (2004)[25]. Cycle parameters included: denaturation at 95°C for 45 sec; 95°C for 0 s, 48°C for 0 s, 72°C for 30 cycles; final extension at 72°C for 1 min. PCR products were then run on a 1% agarose gels stained with gel red and successful samples were gel extracted using Qiagen's gel extraction kit following the manufacturer's instructions for DNA purification for sequencing. Samples were submitted to Quintara biosciences for sequencing. Data were examined in ApE to determine the sequence at codon 96 and to look for evidence of heterozygosity. Sequence was also translated in ExPatsy to confirm the identity of codon 96.

Real-Time Quaking Induced Conversion (RT-QuIC)

RT-QuIC analysis was performed on some isolates that did not give interpretable western blot data to verify that the samples had seeding activity and were simply below the limit of detection by our assays. Samples were processed by RT-QuIC as described by Denkers *et al* (2020)[26]. Briefly, 2 µl of sample diluted to 10^{-5} in 0.1% SDS was added to 96 µl of substrate (0.10 mg/ml rPrP, 10 µM thioflavin T (ThT), 320 mM NaCl, 1mM EDTA and 1X PBS and processed for a total of 62.5 h at 42°C on a shaking fluorimeter as described[26]. At least 8 replicates for each sample were run over two separate reaction plates. Data are shown as 1/lag phase to represent the rate of the reaction. The lag phase is described as the time (in hours) it takes the sample fluorescent signal to cross the fluorescent signal of 5x the standard deviation of the average baseline fluorescence[26].

Data analysis

Densitometric analyses for conformational stability and glycoform ratio were completed in ImageJ. Statistical analysis and graphing were performed in GraphPad Prism (version 8.30). Conformational stability was determined by calculating the concentration at which the signal was half of the input ($[GndHCl]_{1/2}$) after fitting the data to a fourth order polynomial regression. If the sample crossed the 50% line more than once, each [GndHCl] at which the signal dropped below 50% was included in the calculation of the average $[GndHCl]_{1/2}$. Average $[GndHCl]_{1/2}$ values were calculated by determining the [GndHCl] that was required for there to be 50% of the original signal detected for each replicate and taking the average and standard deviation of the replicates. Graphs were generated by averaging the signal across concentrations. This distinction is important because of the sizable differences that were

observed between calculating the average of the $[GndHCl]_{1/2}$ of each sample or graphing an average denaturation curve and calculating a $[GndHCl]_{1/2}$.

Glycoform ratio was calculated in ImageJ by determining what percentage of the total signal was contributed by each glycosylation state. Glycoform ratio data was arcsine transformed before analysis so percent data would fit a normal distribution and could be compared statistically. Comparisons were made only between equivalent band types. For example, the diglycosylated band of one sample was only compared to the diglycosylated band from the other isolates. Different bands (e.g., di- and monoglycosylated) were not compared. Glycoform ratio triplots were constructed in JMP Pro 15.

Only samples that had at least 2 successful replicates from at least three experimental attempts (conformational stability) or had results replicated on at least two blots with two samples each (glycoform ratio) were included for analysis so that only data that we reproducible was utilized. For comparisons involving more than 2 comparisons, an ANOVA was run with a Tukey adjustment for multiple comparisons (Prism). For comparison between 2 groups, an unpaired t-test was performed (Prism).

Principle component analysis

To visualize the data in a way that incorporates multiple biochemical strain features as well as sex, age, genotype and location information, principle component analysis (PCA) was performed using ClustVis[27]. Unit variance scaling was applied to rows and singular value decomposition with imputation was used to calculate principal components. Within-site comparisons were conducted if location information was available and between site comparisons were conducted and separated either by location or tissue type.

Results

Sample distribution & submission

Samples were submitted from Michigan, Missouri, Arkansas, Virginia and Iowa (Figure 3.1). Isolates from each state were assessed for both within and between site differences. Within site differences will be discussed first, followed by an assessment of between-state differences.

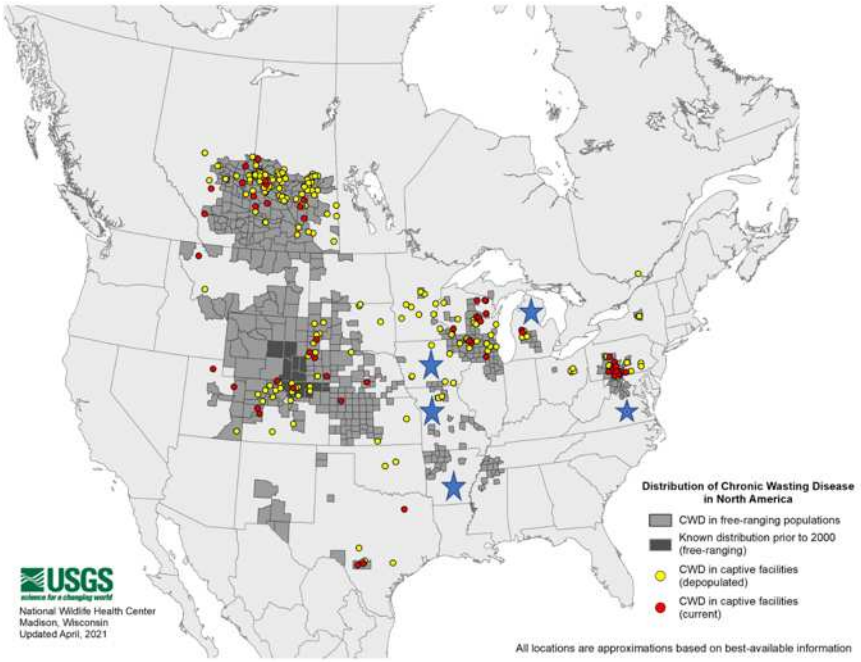

Figure 3.1. CWD distribution in North America. Map of CWD cases is from USGS. States that submitted samples for strain typing analysis are noted with blue stars. Stars were placed on states such that none of the known free-ranging or captive case distribution were blocked from view.

Michigan

In total, 13 retropharyngeal lymph node samples from CWD-positive, free-ranging white tailed deer were submitted for strain-typing analysis (Table 3.1). In addition to the strain typing information gathered as the result of these studies, haplotype, genotype, sex and age information of these animals were provided (Table 3.1) as well as geographical distribution (Figure 3.2). Samples were grouped into two groups based on distribution of the samples (Figure 3.2).

Table 3.1. Isolate identification, description and result summary for white-tailed deer LNs from MI, USA.

Sample ID	Glycoform Ratio	CSA*	Genotype	Haplotype 1^	Haplotype 2^	SEX	AGE (Years)	Location
583574	Yes	Yes	96GG	A	A	male	1	1
576725	Yes	Yes	96GG	A	A	male	2	1
575004	Yes	Yes	96GG	B	B	male	2	1
506421	Yes	Yes	96GG	A	A	male	3	2
580049	Yes	Yes	96GG	B	B	male	3	1
567818	Yes	Yes	96GG	A	A	male	1	1
578385	Yes	Yes	96GG	A	B	female	1	1
508792	Yes	Yes	96SS	C	C	male	1	1
576801	Yes	Yes	96GG	A	E	female	3	1
421858	Yes	No	96GG	A	O	female	3	2
565264	Yes	No	96GG	A	A	male	1	1
507461	Yes	No	96GG	D	B	female	2	2
455702	No	No	96GG	B	B	male	1.5	2

*Conformational stability assay
^Haplotypes description as in Brandt *et al.* (2015)[28]

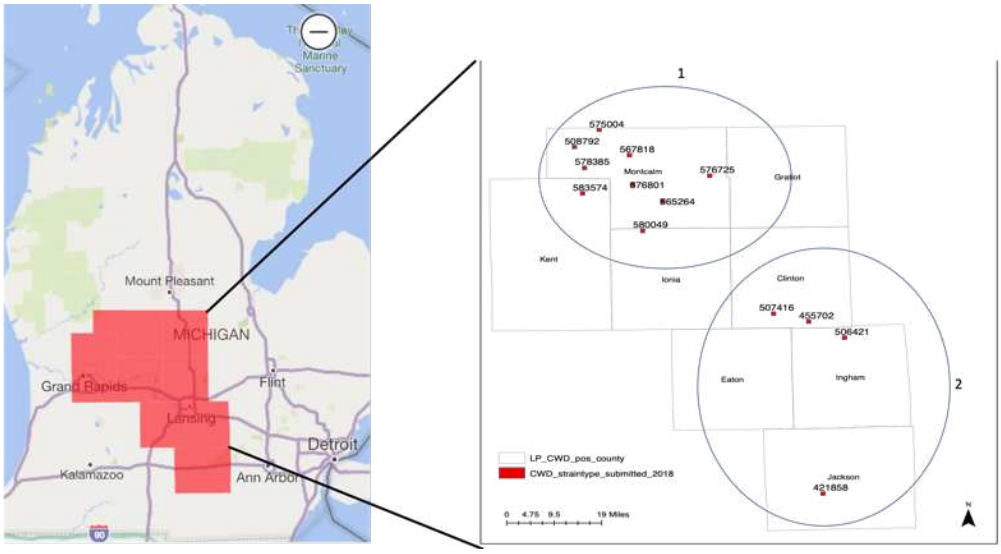

Figure 3.2. Geographical distribution of from CWD-positive white tailed deer lymph nodes isolates from Michigan, USA. A map showing the distribution of samples used for CWD strain typing from MI. To give an idea of the location of the counties in Michigan, CWD positive counties are highlighted in red on a map of lower Michigan. Insert shows more detailed submitted sample distribution. Samples were grouped into groups 1 and 2 based on geographical proximity. Michigan map was taken from the Michigan DNR's website on CWD distribution[29].

Conformational stability of the individual isolates was determined as described in the methods section. One of the goals of this project was to determine if there were any differences within a site. For that reason, the results for each individual animal are shown separately (Figure 3.3). When looking at the shape of the curves, there are three general shapes that emerge: 1) some samples have a 4th order polynomial curve that looks like standard denaturation curves for CWD (Figure 3.3 A, B, D, H), 2) another curve shape appears to drop off more steeply and immediately (Figure 3.3, F, I), and 3) samples that appear to "smile", where they cross (or nearly cross) the 50% loss of the total signal on the 4th order polynomial regression twice (Figure 3.3 C, E, G). No statistical differences were found between the mean $[GndHCl]_{1/2}$ between any of the isolates (Figure 3.4).

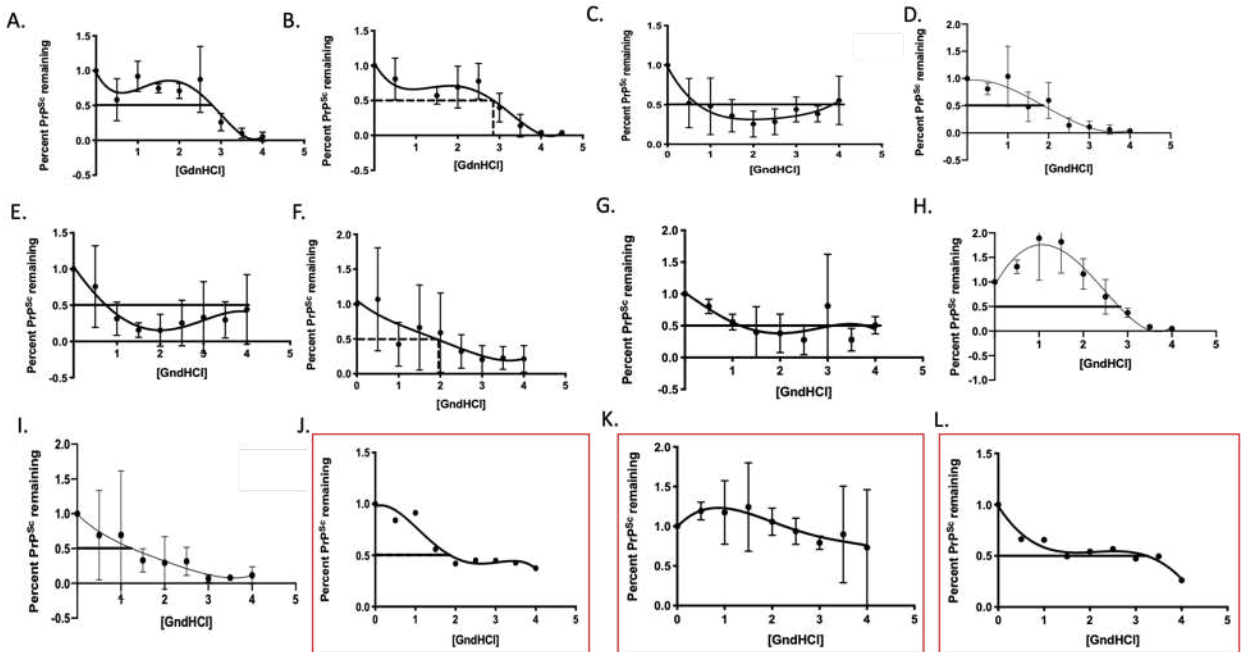

Figure 3.3. Denaturation curves for individual isolates from Michigan, USA show three unique shapes. Conformational stability curves for MI isolates 578385 (A), 567818 (B), 508792 (C), 576725 (D), 506421 (E), 575004 (F), 580049 (G), 583574 (H), 567801 (I), 421858 (J), 507416 (K), 565264 (L) are shown. Graphs show the average of at least two successful replicates from three independent experiments. Lines on the graph indicate the point at which only 50% of the original signal is still detected. The samples highlighted in red are shown but were not included in any further analyses because only 1 replicate gave interpretable signal (J, L) or because the signal never went below 50% of the starting signal (K). Graphs show the mean and standard deviation at each [GndHCl].

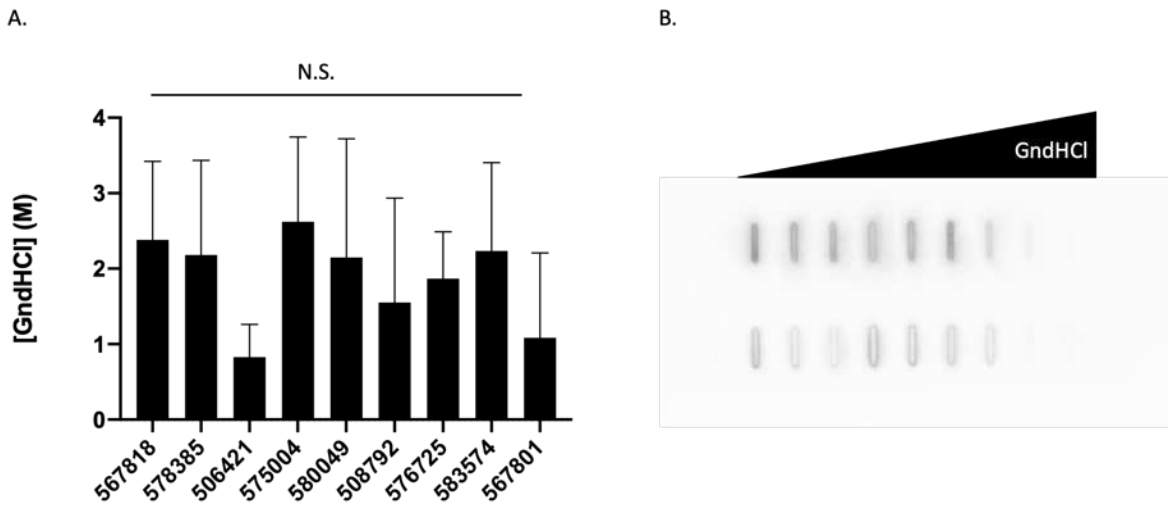

Figure 3.4. There are no significant differences in $GndHCl_{1/2}$ between MI isolates. Replicates for each isolate were graphed individually and the $GndHCl_{1/2}$ for each replicate was determined and averaged together to calculate a mean (A). The mean of each isolate represents at least two successful replicates from at least three independent attempts. Graph shows mean ± standard deviation Representative slot blot image is also shown (B). There are no statistical differences between any of the isolates ($p>0.05$). One-way ANOVA with Tukey adjustment. N.S., not significant.

Isolates were then assessed for glycoform ratio and compared to determine if there were any statistical differences in the percent of the total signal represented by each glycosylation state (di-, mono-, or unglycosylated; Figure 3.5). While every isolate was predominately diglycosylated, which is expected in CWD prions, one isolate (506421) had di- and mono-glycosylated bands that contributed a more equal amount to the total signal. Indeed, there were significant differences between 506421 and five MI isolates (Figure 3.5, Table 3.2). The other samples had the diglycosylated form of the prion contributing to the majority of the signal.

While collecting glycoform ratio data, a unique electrophoretic pattern was noted where there appeared to be a doublet in the diglycosylated band some of the time when the blots ran well (Figure 3.6). To determine if this was the result of alternative PK digestion sites, samples were treated with PNGase F to remove the glycan trees so any differences in protein size could be observed. No doublet was observed after PNGase F treatment, indicating the doublet was not the result of alternative digestion sites but is perhaps the results of alternative glycan trees or interference with another protein (Figure 3.6).

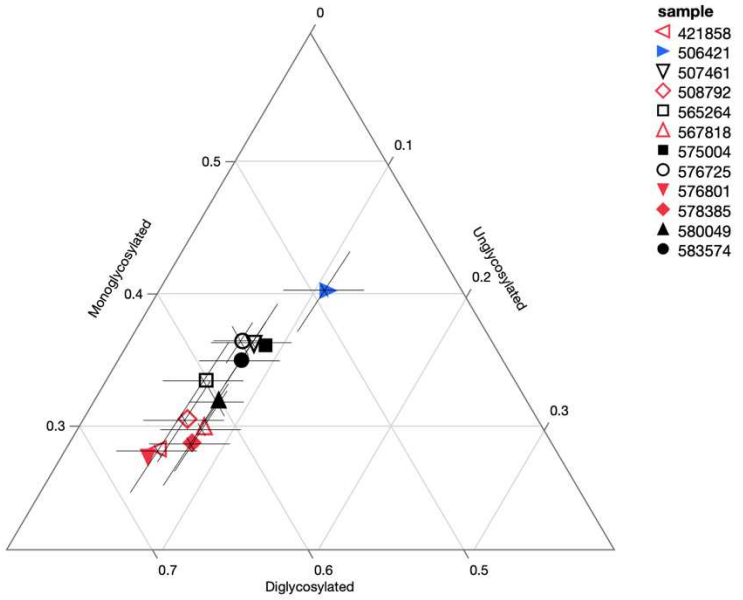

Figure 3.5. Isolate 506421 is significantly different from five other MI isolates. Significant differences are found between MI isolate 506421 (blue) and 5 other MI isolates (red) in the di- and mono- glycosylated bands. There are no other significant differences between any other samples. One way ANOVA with Tukey adjustment. Significant differences are summarized in Table 3.2.

Table 3.2. Summary of significant differences in glycoform ratio between MI Isolates.

Sample ID	Diglycosylated		Monoglycosylated	
	Summary	Adjusted p-value	Summary	Adjusted p-value
506421 vs. 578385	**	0.004	**	0.0086
506421 vs. 567818	**	0.0088	*	0.0203
506421 vs. 421858	***	0.0003	**	0.0044
506421 vs. 508792	***	0.0006	*	0.0102
506421 vs. 576801	***	0.0003	**	0.0053

* p<0.05, **p<0.01, ***p<0.001

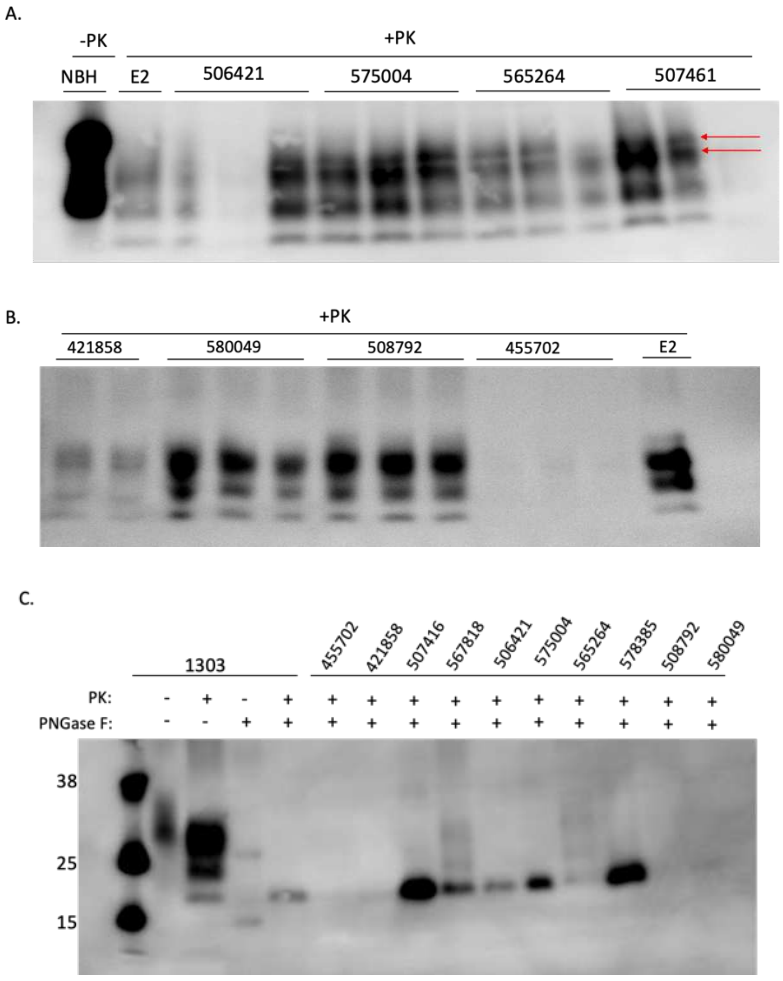

Figure 3.6. MI isolates have a unique electrophoretic pattern that is not the result of alternative PK digestion sites. While running western blots to obtain glycoform ratio data, it was noted that the diglycosylated band had two discrete bands noted by the red arrows (A). This pattern was observed in the samples when the gel ran well but was not noted when the band didn't run quite as well, as shown in B. PNGase F treatment showed that all the samples collapsed to a single band, indicating there are not alternative PK digestion sites because they collapse to the same size as the control sample (1303) when treated with both PK and PNGase F and this control did not have evidence of two discrete diglycosylated bands (C). 1303 is a lymph node sample from an experimentally infected white-tailed deer, a gift from Dr. Hoover's lab.

To determine if isolates (N=12) would cluster or any trends could be observed based on the results of the biochemical strain typing analysis, principle component analysis (PCA) was used. Samples were grouped by location and symbols represent the sex of the animals. Age, haplotype, glycoform ratio and conformational stability were incorporated in the construction of the PCA. Discrete variables (i.e., haplotype) were converted to continuous variables so they could be incorporated in the construction of

the PCA. There was no clear separation between location 1 and 2 but isolate 506421 did separate from the other isolates from Michigan (Figure 3.7).

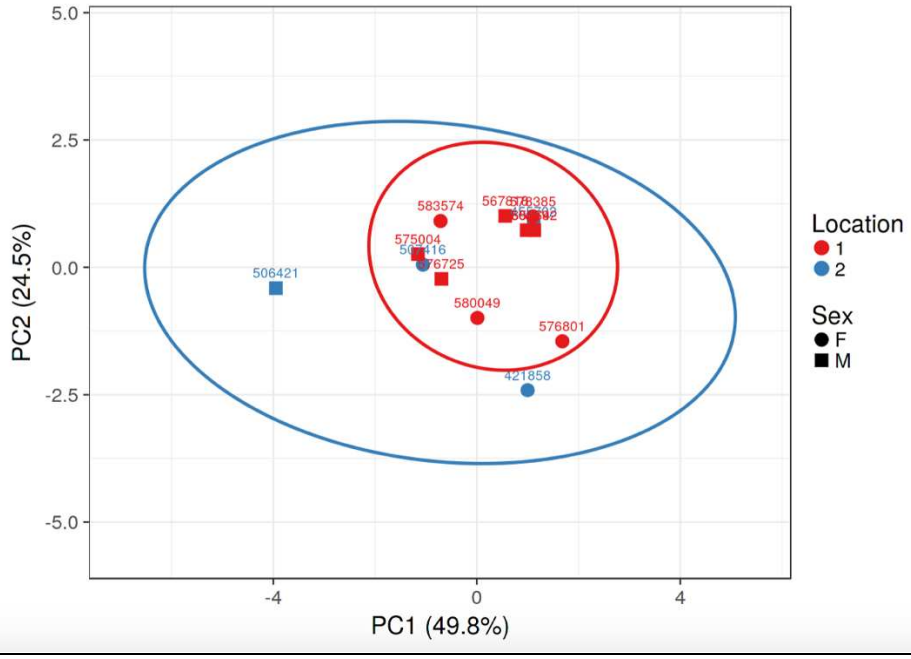

Figure 3.7. Principle Component Analysis of MI isolates do not show distinct clustering between location 1 and location 2 from MI but isolate 506421 groups separately from the other isolates. Conformational stability, glycoform ratio, haplotype and age information were used to calculate the PCA. Categorical variables were converted to numerical values so they could be included in the analysis. X and Y axis show principal component 1 and principal component 2 that explain 40.9% and 18.7% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 12 data points[27]. There is substantial overlap between the two locations but isolate 506421 groups separately from the other isolates.

Missouri

A total of 16 retropharyngeal lymph node samples from Missouri were assessed for biochemical prion strain typing analysis and genotyping; results are summarized in Table 3.3. A map showing the distribution of the isolates was also provided (Figure 3.8).

Table 3.3. Sample identification, description and result summary for white-tailed deer lymph node isolates from Missouri, USA.

Sample ID	Glycoform ratio	CSA	Genotype	Location	Condensed Location	Inoculated for Bioassay?
20064513	Yes	Yes	96GG	2	1	Yes
20062199	Yes	Yes	96GG	3	1	No
20074043	Yes	Yes	96GG	5	3	No
20075259	Yes	Yes	96GG	5	3	Yes
20074689	Yes	Yes	NA	6	3	Yes
20080051	Yes	Yes	96GG	7	3	Yes
20053581	Yes	Yes	96GG	4	2	Yes
20058516	Yes	Yes	NA	4	2	No
20067133	Yes	Yes	96GG	1	1	Yes
20084873	Yes	Yes	96GG	4	2	Yes
20062466	Yes	Yes	96GG	2	1	No
20074072	Yes	Yes	96GG	5	3	No
20063058	Yes	No	96GG	3	1	No
20062501	Yes	Yes	96GG	3	1	Yes
20066808	Yes	Yes	NA	3	1	No
20080215	No	No	96GG	7	3	No

NA = not available, samples did not amplify in PCR for submission for sequencing.
CSA = conformational stability assay

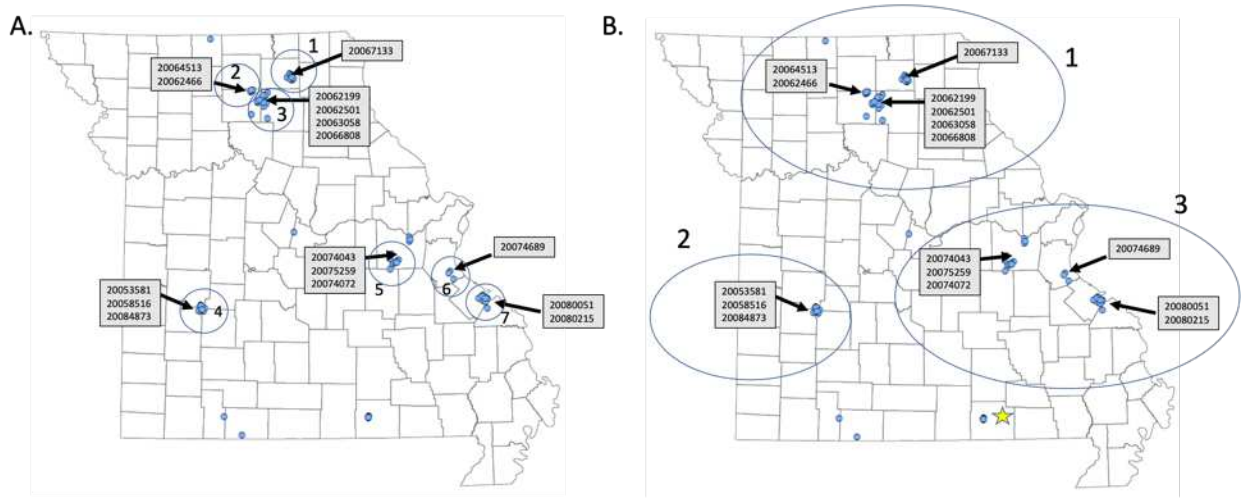

Figure 3.8. Geographical distribution of Missouri white-tailed deer isolates. Samples were compiled into 7 groups (A) or 3 groups (B) based on geographical distribution of CWD outbreaks. The location designations were incorporated into the construction of PCA for within-state comparisons.

To determine if there were any within state differences in conformational stability, individual isolate denaturation curves are shown (Figure 3.9). As with the isolates from MI, there appear to be different curve shapes. About half the samples drop below 50% of the original starting signal at about 1 M GndHCl (Figure 3.9 C, D, G, I, J, K) and the other half of the samples have higher $GndHCl_{1/2}$ and the samples decrease in signal less rapidly (Figure 3.9 A, B, E, F, H, L, M). The difference in the curve shape is related to where the samples drop below 50% of the original starting concentration, which should be reflected in the average $[GndHCl]_{1/2}$. There were no significant differences between the mean $[GndHCl]_{1/2}$ between any of the samples (Figure 3.10, one-way ANOVA with Tukey's multiple comparison, $p>0.05$). While there weren't any significant differences, 20026466 has a much lower mean $GndHCl_{1/2}$ (Figure 3.9). The low sample size may explain why the comparisons were not significant.

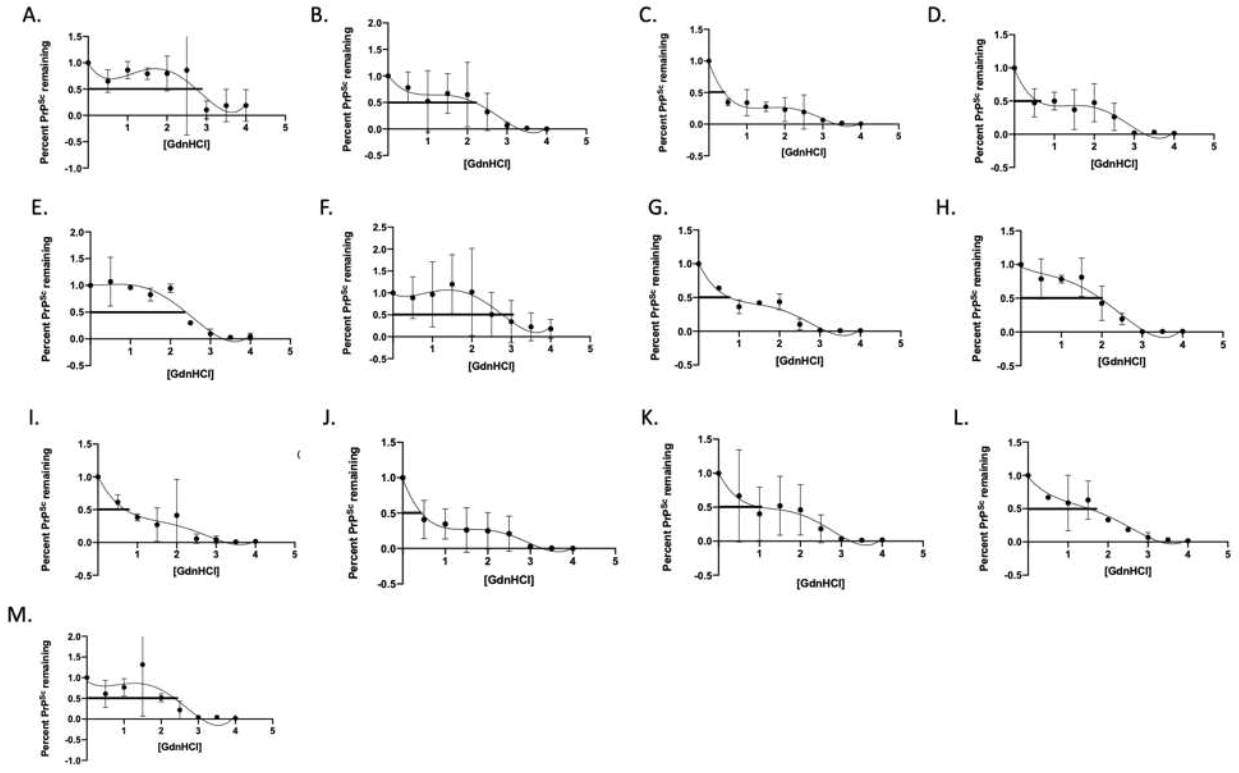

Figure 3.9. Denaturation curves for CWD isolates from Missouri, USA. Conformational stability curves for MO isolates 20053581 (A), 20062199 (B), 20026466 (C), 20067133 (D), 20064513 (E), 20074043 (F), 20074072 (G), 20066808 (H), 20080051 (I), 20075259 (J), 20084873 (K), 20062501 (L), 20074689 (M). Graphs show the average of at least two successful replicates from three independent experiments. Lines on the graph indicate the point at which only 50% of the original signal is still detected.

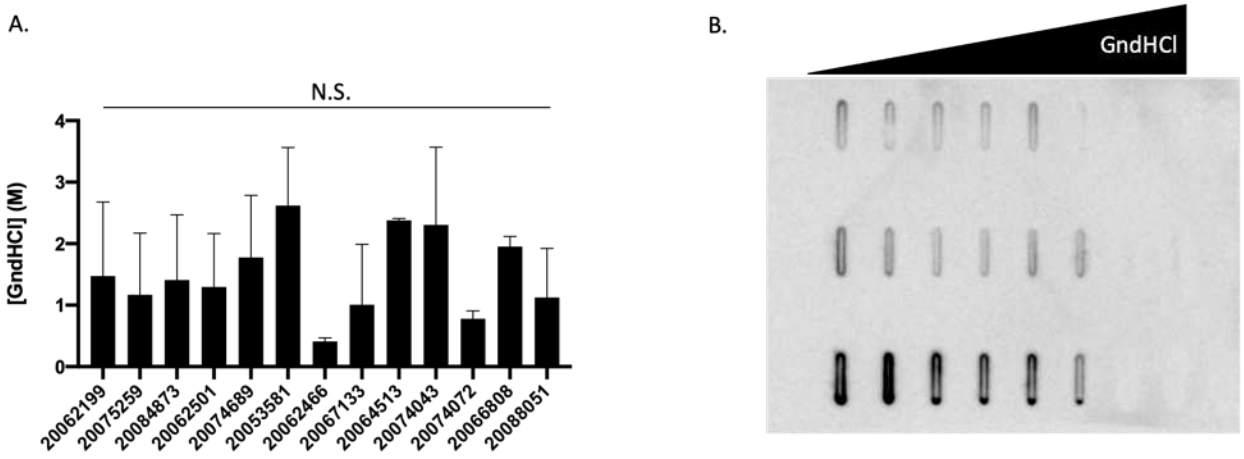

Figure 3.10. There are no differences in the mean $[GndHCl]_{1/2}$ between any of the isolates from Missouri. Mean and standard deviation for the $[GndHCl]_{1/2}$ are shown (A). Representative slot blot is shown. GndHCl concentration increases from 0 M to 4 M in 0.5 M increments (B). No statistical differences were found between any of the samples. One-way ANOVA with Tukey adjustment. N.S. = not significant.

To continue to examine the differences between isolates from Missouri, samples were compared by glycoform ratio (Figure 3.11) and numerous differences were found (summarized in Table 3.4). While all of the isolates have primarily diglycosylated PrP^{Sc}, there are some isolates that have diglycosylated and monoglycosylated bands that make up closer to an equal proportion of the total signal than samples that have substantially more diglycosylated PrP^{Sc}.

To incorporate all the biochemical strain typing information, as well as location and condensed location to see if there was any clear clustering of samples a PCA was performed (Figure 3.12). Samples were clustered based on location (Figure 3.12 A) or condensed location (Figure 3.12 B). There was no clear clustering of groups based on location, but there was tighter grouping in samples from condensed location 3 (Figure 3.12 B).

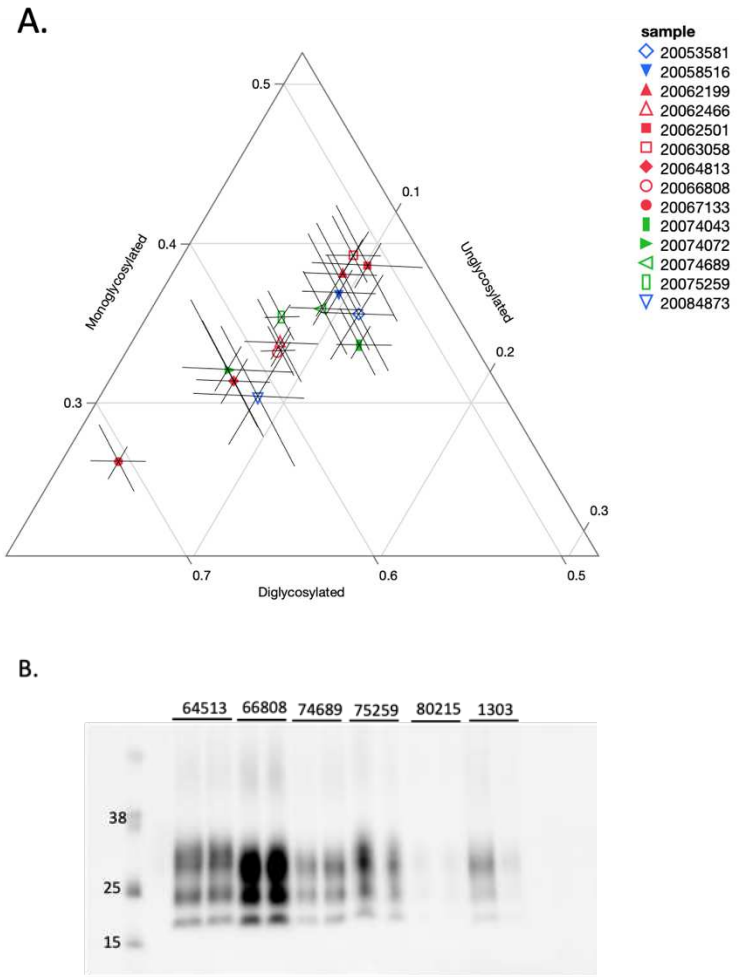

Figure 3.11. There are significant differences in glycoform ratio between free-ranging white tailed deer isolates from Missouri, USA. The amount that each di-, mono- and unglycosylated bands contribute to the total signal are graphed as a triplot (A). Symbols in red, blue and green represent the animals that were from condensed locations 1, 2 or 3, respectively. There were many significant differences found between individual isolates and the significant differences are summarized in Table 3.4. Representative glycoform ratio blot is shown in B. For the ease of labeling, the 200- at the front of each isolate ID was dropped. 1303 is a positive control white-tailed deer lymph node that was run as a control.

Table 3.4. Significant differences in glycoform ratio between isolates from MO

Sample ID	Diglycosylated		Monoglycosylated		Unglycosylated	
	Summary	Adjusted P Value	Summary	Adjusted P Value	Summary	Adjusted P Value
53581 vs. 74072	ns	0.0944	ns	0.8354	*	0.0332
53581 vs. 67133	****	<0.0001	***	0.0004	****	<0.0001
62466 vs. 67133	**	0.0024	*	0.0175	**	0.0085
64813 vs. 62199	ns	0.0682	*	0.0410	ns	0.9884
64813 vs. 63058	*	0.0111	**	0.0031	ns	0.7930
64813 vs. 62501	**	0.0063	*	0.0112	ns	0.3418
74072 vs. 63058	*	0.0181	*	0.0170	ns	0.5167
74072 vs. 74043	ns	0.4720	ns	>0.9999	*	0.0223
74072 vs. 62501	*	0.0107	ns	0.0501	ns	0.1518
74689 vs. 67133	****	<0.0001	****	<0.0001	****	<0.0001
62199 vs. 84873	ns	0.1559	**	0.0084	ns	>0.9999
62199 vs. 67133	****	<0.0001	****	<0.0001	**	0.0032
58516 vs. 84873	ns	0.2326	*	0.0456	ns	0.9973
58516 vs. 67133	****	<0.0001	****	<0.0001	****	<0.0001
63058 vs. 84873	*	0.0351	***	0.0005	ns	>0.9999
63058 vs. 67133	****	<0.0001	****	<0.0001	***	0.0002
74043 vs. 67133	****	<0.0001	ns	0.0715	****	<0.0001
75259 vs. 67133	***	0.0003	***	0.0005	*	0.0202
84873 vs. 67133	ns	0.0780	ns	0.7896	**	0.0079
84873 vs. 62501	*	0.0214	**	0.0018	ns	0.9708
67133 vs. 66808	**	0.0030	*	0.0368	**	0.0031
67133 vs. 62501	****	<0.0001	****	<0.0001	****	<0.0001

ns = not significant, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

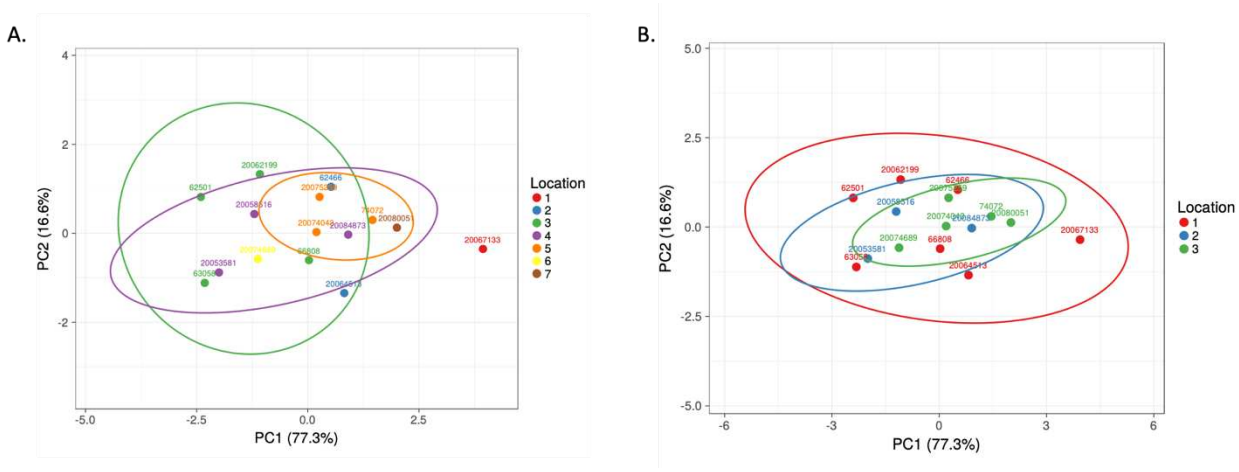

Figure 3.12. No clear separation of CWD isolates from MO by location. Conformational stability, glycoform ratio and location information were incorporated into the PCA. There is substantial overlap between the locations (A) and condensed locations (B), with the greatest variance in condensed location 1 (B). X and Y axis show principal component 1 and principal component 2 that explain 16.6% and 77.3% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse[27].

Arkansas

Samples from Arkansas were used to compare between the brain and lymph node of the same individual animal (discussed in Chapter 2). Individual denaturation curves, comparison of mean $GndHCl_{1/2}$ values, and glycoform ratio data are shown in Chapter 2. Within site differences from Arkansas were discussed in Chapter 2 and data will not be shown again here but will be discussed and incorporated in the between site comparisons.

Virginia

Fourteen lymph nodes from white-tailed deer were submitted for strain typing analysis from Virginia, USA. Of the 14 samples that were submitted, 9 samples had interpretable data by both glycoform ratio and conformational stability assessment (Table 3.5). To compare isolates within Virginia by conformational stability, individual denaturation curves are all graphed for individual isolates are shown in Figure 3.13. The denaturation curves all look very similar for these isolates as opposed to the isolates tested from MI and MO where a lot of variability was observed. There were no significant differences in the mean $[GndHCl]_{1/2}$ between any of the isolates from Virginia (Figure 3.14).

Table 3.5. Sample identification, description and result summary for white-tailed deer lymph node isolates from Virginia, USA.

Sample ID	Glycoform Ratio	CSA
B26907	Yes	Yes
A12448	Yes	Yes
A11143	Yes	Yes
A1003	Yes	Yes
T01615	Yes	Yes
B26052	Yes	Yes
B21336	Yes	Yes
T02033	Yes	Yes
B21416	Yes	Yes
A13187	No	No
A13183	No	No
A11692	No	No
B25491	No	No
A13219	No	No

CSA= conformational stability assay

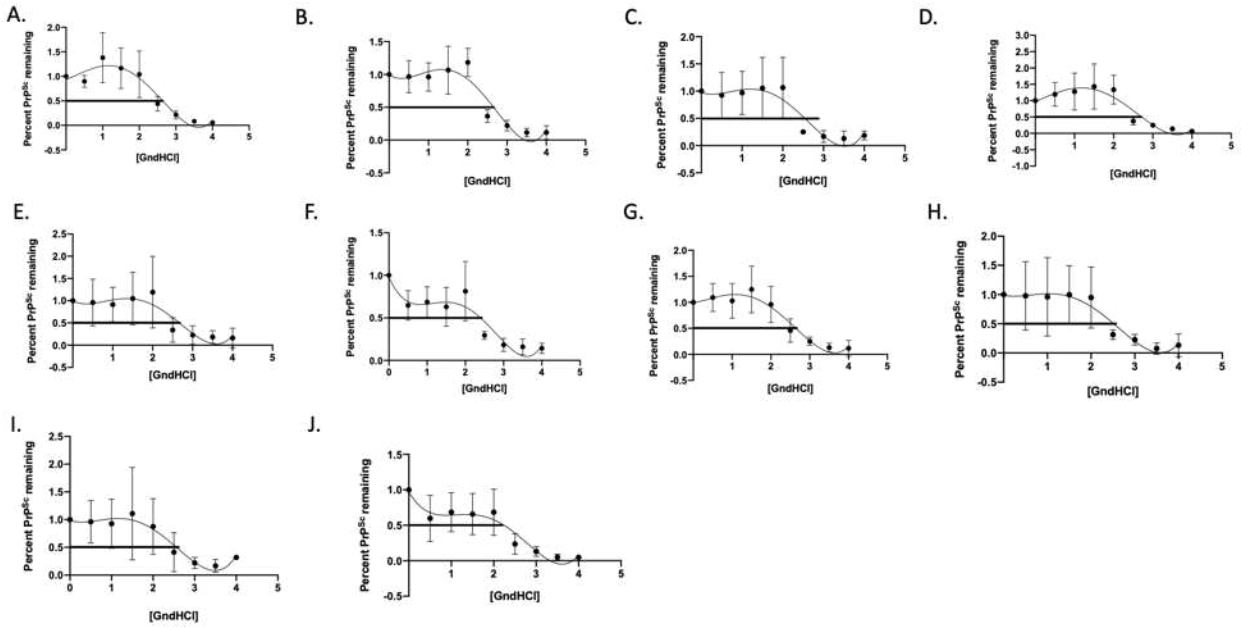

Figure 3.13. Denaturation curves for CWD isolates from Virginia, USA, show stable, classical CWD in all isolates. Conformational stability curves for VA isolates B26907 (A), A12448 (B), A11143 (C), A11003 (D), A11858 (E), B21336 (F), T02033 (G), B21416 (H), B26032 (I). Graphs show the average of at least 3 successful replicates. Average and standard deviation of each point were fit to a 4th order polynomial regression. Lines on the graph indicate the point at which only 50% of the original signal is still detected.

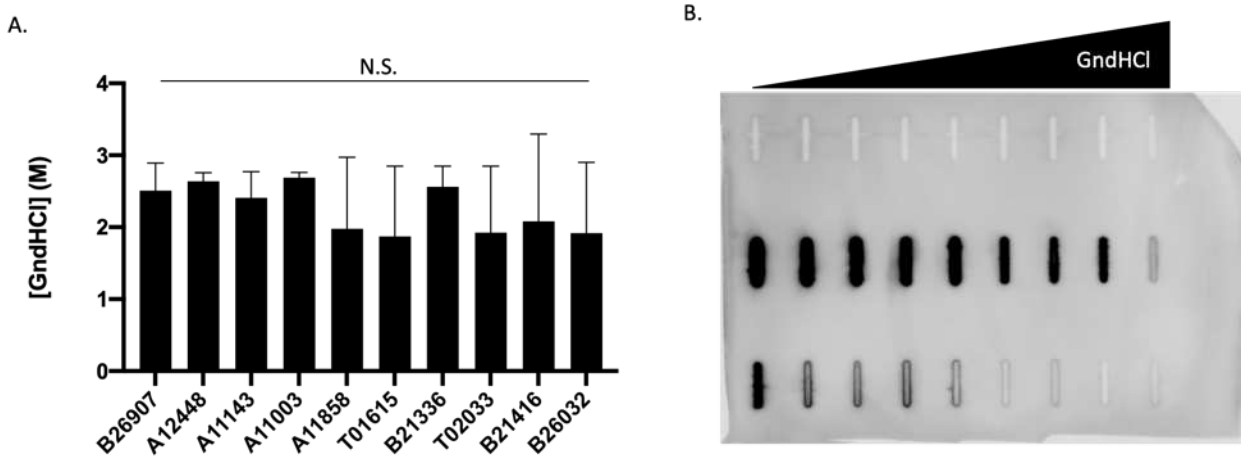

Figure 3.14. No differences in [GndHCl]$_{1/2}$ between individual white-tailed deer isolates from Virginia, USA. Mean and standard deviation for the [GndHCl]$_{1/2}$ are shown (A). Representative slot blot is shown (B). No statistical differences were found between any of the samples. One-way ANOVA with Tukey adjustment. N.S. = not significant.

Glycoform ratio differences were also found between many of the isolates from Virginia (Figure 3.15, Table 3.6). While differences were found, all of the isolates are primarily diglycosylated, as expected for classical CWD. Because there is no location information or additional data to separate the isolates at this time, PCA was not attempted to look at within site differences.

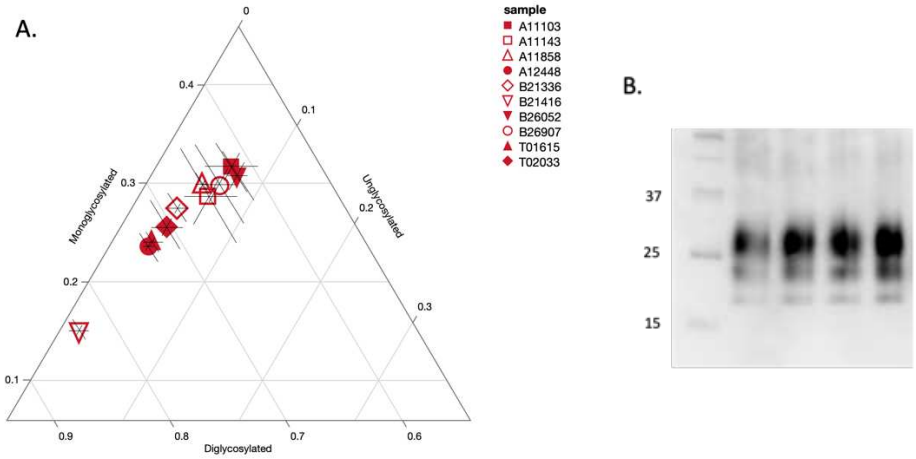

Figure 3.15. Glycoform ratio between white-tailed deer isolates from Virginia, USA, have many significant differences. The amount that each di-, mono- and unglycosylated bands contribute to the total signal are graphed as a triplot (A). There were many significant differences found between individual isolates and the significant differences are summarized in Table 3.6. Representative glycoform ratio blot is shown in B.

Table 3.6. Significant differences in glycoform ratio between white-tailed deer isolates from Virginia, USA.

Sample Comparison	Diglycosylated		Monoglycosylated		Unglycosylated	
	Summary	Adjusted P Value	Summary	Adjusted P Value	Summary	Adjusted P Value
B26907 vs. A12448	*	0.0328	*	0.0170	ns	0.4005
B26907 vs. B21416	****	<0.0001	****	<0.0001	*	0.0441
A12448 vs. A11003	**	0.0023	***	0.0002	ns	0.2338
A12448 vs. A11858	ns	0.1071	**	0.0098	ns	0.9446
A12448 vs. B21416	*	0.0389	**	0.0012	ns	0.8894
A11143 vs. B21416	****	<0.0001	****	<0.0001	ns	0.1909
A11003 vs. T01615	*	0.0208	**	0.0059	ns	0.3611
A11003 vs. T02033	*	0.0392	*	0.0122	ns	0.4065
A11003 vs. B21416	****	<0.0001	****	<0.0001	*	0.0224
A11858 vs. B21416	****	<0.0001	****	<0.0001	ns	0.2604
T01615 vs. B21416	*	0.0474	**	0.0013	ns	0.9389
B26052 vs. B21416	****	<0.0001	****	<0.0001	*	0.0316
B21336 vs. B21416	***	0.0003	****	<0.0001	ns	0.6322
T02033 vs. B21416	**	0.0031	****	<0.0001	ns	0.7291

ns = not significant, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Iowa

Nine samples from Iowa, USA were submitted for biochemical strain typing analysis and four samples gave interpretable data for analysis (Table 3.7). Individual denaturation curves are shown for each of the four isolates and the general shape of the curves looks the same for all four isolates (Figure 3.16). There were no significant differences in stability between any of the isolates (Figure 3.17).

Table 3.7. Sample ID and result summary from animals from Iowa, USA.

Sample ID	Glycoform Ratio	CSA
193793	Yes	Yes
191058	Yes	Yes
195088	Yes	Yes
197186	Yes	Yes
070	No	No
197186	No	No
198393	No	No
193900	No	No
C603	No	No

CSA=conformational stability assay

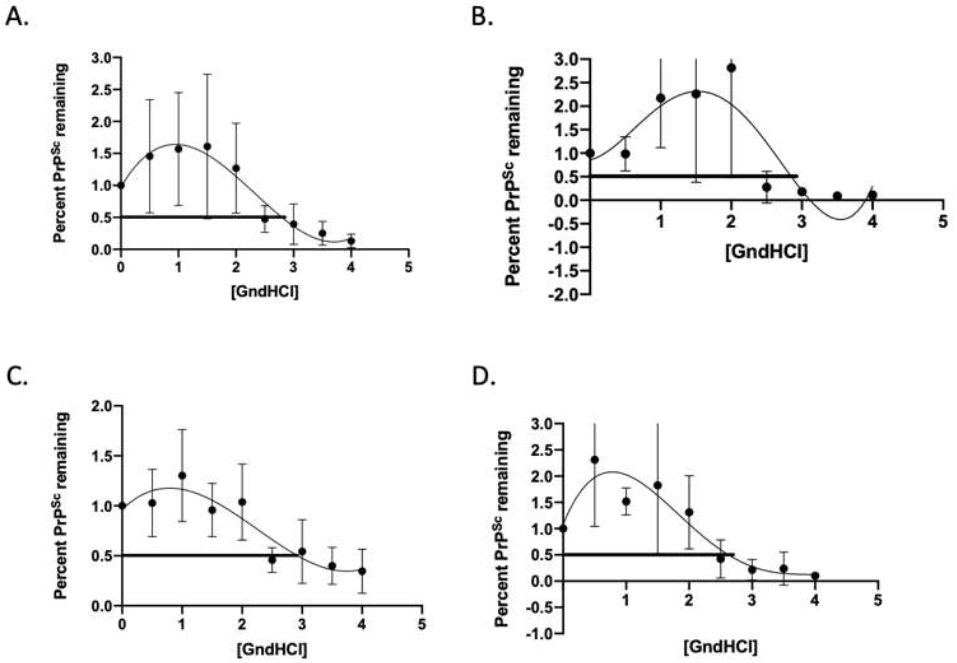

Figure 3.16. Denaturation curves for individual isolates from Iowa, USA, demonstrate stable, classical CWD. Samples shown are 193793 (A), 191058 (B), 197186 (C), 195088 (D). Denaturation curves for graphs show the average of at least 3 successful replicates. Average and standard deviation of each point were fit to a 4th order polynomial regression. Lines on the graph indicate the point at which only 50% of the original signal is still detected.

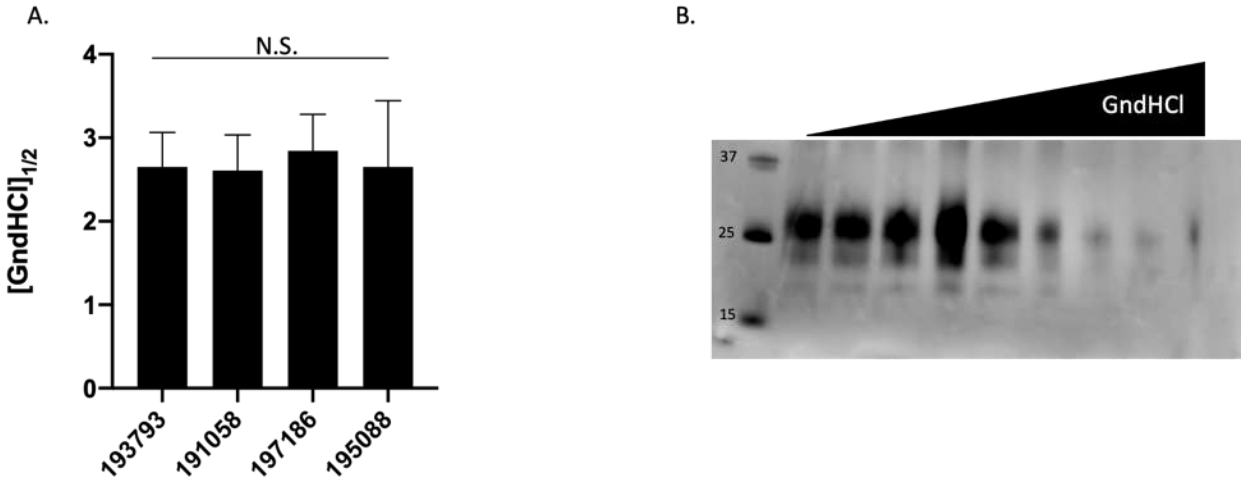

Figure 3.17. No difference in mean $[GndHCl]_{1/2}$ between any of the isolates from Iowa, USA. Mean and standard deviation for the $[GndHCl]_{1/2}$ are shown (A). Representative denaturation western blot is shown (B). No statistical differences were found between any of the samples. One-way ANOVA with Tukey adjustment. N.S. = not significant.

When comparing isolates from Iowa by glycoform ratio, it was apparent that all of the samples were predominately diglycosylated as expected for CWD prions. There was only one statistical difference between isolate 197186 and isolate 191058 in the unglycosylated band (Figure 3.18). With so few samples and no additional location information, PCA was not run on these samples for within site comparisons.

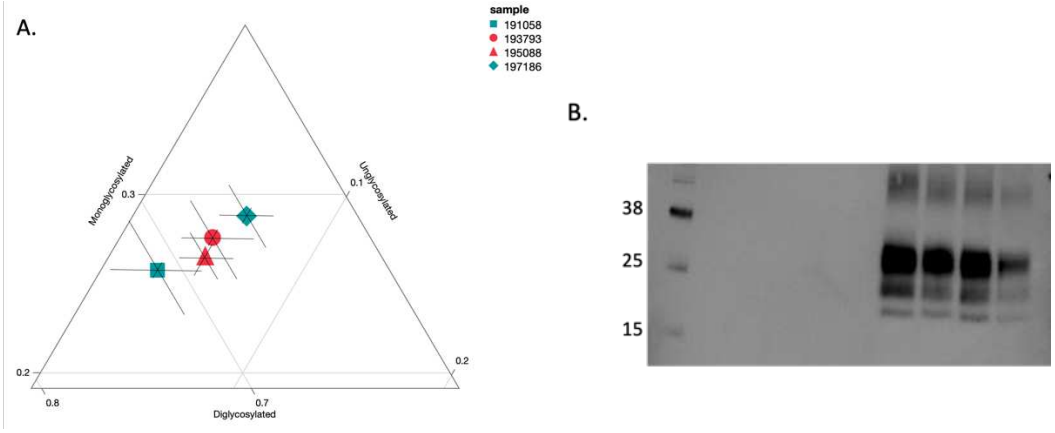

Figure 3.18. Significant differences in glycoform ratio between two isolates from Iowa, USA. Triplot showing percentage of the total signal that was contributed by each individual glycosylation state (A). Representative western blot showing an unsuccessful sample and a successful sample that was used to calculate glycoform ratios (B). The only statistical difference between any of the samples was in the unglycosylated band when compared between sample 191058 and 197186 (blue symbols). Points show the mean and error bars indicate standard deviation. One-way ANOVA with Tukey adjustment, $p<0.01$.

USDA

Samples from captive white-tailed deer were submitted for strain typing analysis by the USDA from numerous deer farms across the USA (Table 3.8). Of the nineteen samples that were submitted for strain typing analysis, only 3 had enough detectable signal for CSA and glycoform ratio analysis (Table 3.8). While the samples were received blinded and information as to the positivity of the samples was not received until after the analysis was completed, it was surprising that only three samples had detectable prion signal by western blot. For that reason, samples were assessed for their ability to seed a reaction using real-time quaking induced conversion (RT-QuIC). RT-QuIC revealed that many of the samples demonstrated seeding ability. Samples that would be called positive for seeding under these conditions

gave 8/8 or 6/8 positive replicates. Samples that gave inconclusive results gave either 4/8 or 5/8 positive replicates and negative samples gave 1/8 or 0/8 replicates as positive (Figure 3.19). While there were quite a few samples that gave inconclusive results, these samples are likely still positive and further examination using iron-oxide bead extraction or a lower dilution would give more a more definitive answer as to the positivity of the sample. For the purposes of this study, however, we were satisfied with knowing that there was positivity in at least some of the samples that did not give western blot positivity.

Table 3.8. Sample ID and result summary from animals from deer farms provided by the USDA.

Sample ID	CWD detected?*	Glycoform Ratio	CSA	Sex	Age	Location	Genotype
17-336	Obex & LN	Yes	Yes	F	9 yr	South	GG
19-9	Obex & LN	Yes	Yes	M	2 YR	East	ukn
19-6	Obex & LN	Yes	Yes	F	3 YR	East	ukn
17-368	Obex & LN	No	No	F	4 yr	South	GG
18-19	Obex & LN	No	No	F	8 yr	South	GG
18-57	Obex & LN	No	No	M	8 YR	West	GG
18-58	LN	No	No	M	7 yr	West	GS
18-59	Obex & LN	No	No	M	7 YR	West	GG
18-60	LN	No	No	M	5 YR	West	GS
18-61	Obex & LN	No	No	M	3 YR	West	GG
18-62	Obex & LN	No	No	M	6 YR	West	GS
18-85	Obex & LN	No	No	M	2 YR	East	GS
18-144	Obex & LN	No	No	M	<1yr	East	GS
18-338	ND	No	No	F	7 YR	South	unk
19-4	Obex & LN	No	No	F	6 YR	East	ukn
19-5	Obex & LN	No	No	F	14 YR	East	ukn
19-7	Obex & LN	No	No	F	3 YR	East	ukn
19-8	Obex & LN	No	No	F	4 YR	East	ukn
19-10	Obex & LN	No	No	F	8 YR	East	ukn

*USDA-determined positivty in either the lymph node, obex or both.
ND = none deteced
CSA=conformational stability assay

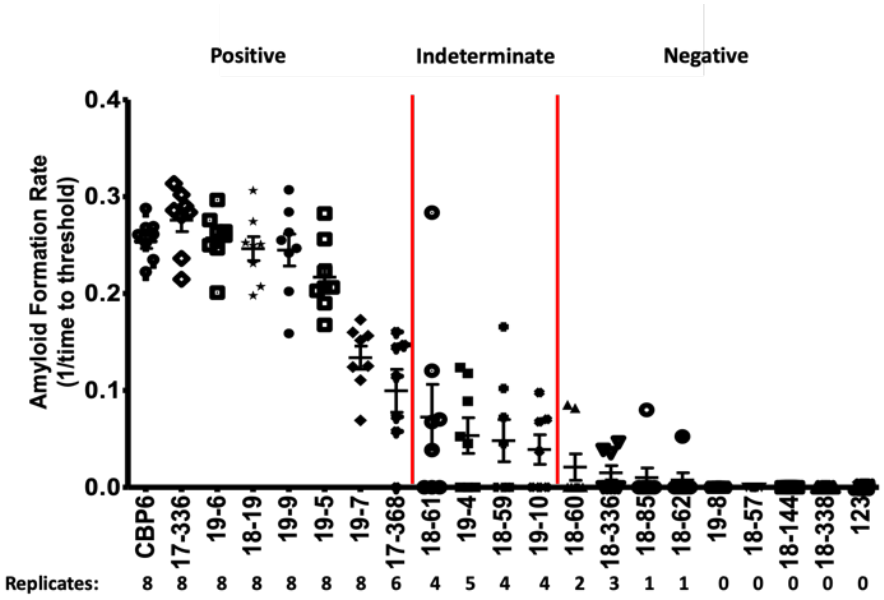

Figure 3.19. RT-QuIC detected some seeding ability in many, but not all, USDA isolates. Samples 17-336, 19-6, 18-19, 19-9, 19-5, 19-7 and 17-368 all had strong, positive seeding activity. Samples 18-61, 19-4, 18-59 and 19-10 all had intermediate positivity that may be positive if additional tests or alternative methods are imposed. Samples 18-85, 18-62, 19-8, 18-57, 18-144, 18-338 are all negative in this experiment. Only isolates 17-336, 19-6 and 19-9 had signal on western blot. CBP6 = cervid brain pool 6, a positive control. 123 = brain from an uninfected white-tailed deer, negative control.

For the samples that did provide interpretable data by western blot, we saw no significant difference in shape of the denaturation curves (Figure 3.20) or average $[GndHCl]_{1/2}$ (Figure 3.21). There were significant differences in glycoform ratio between the unglycosylated band between isolate 17-336 when compared to 19-9 ad 19-6 (Figure 3.22). Interestingly, deer number 17-336 was from a different region than 19-9 and 19-6 (Table 3.8).

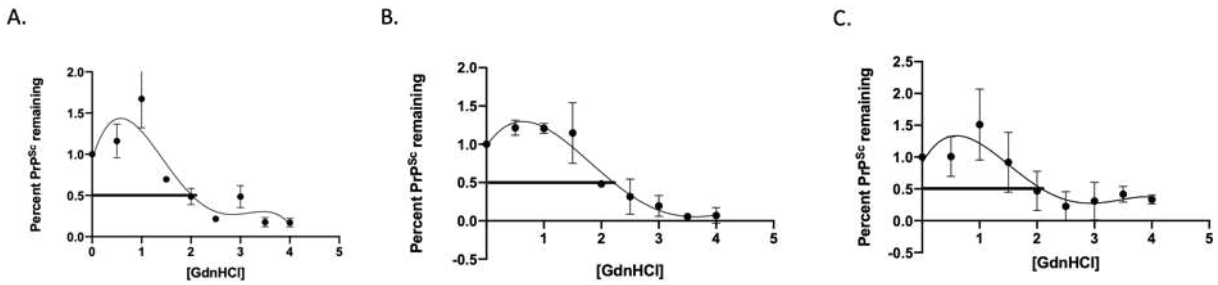

Figure 3.20. Denaturation curves for USDA samples look typical for classical CWD. Denaturation curves for 17-336 (A), 19-6 (B) and 19-9 (C) show the average of at least 3 successful replicates. Curves were fit to a 4th order polynomial regression. Mean and standard deviation at each [GndHCl] are shown. Solid lines on the graph indicate the point at which only 50% of the original signal is still detected.

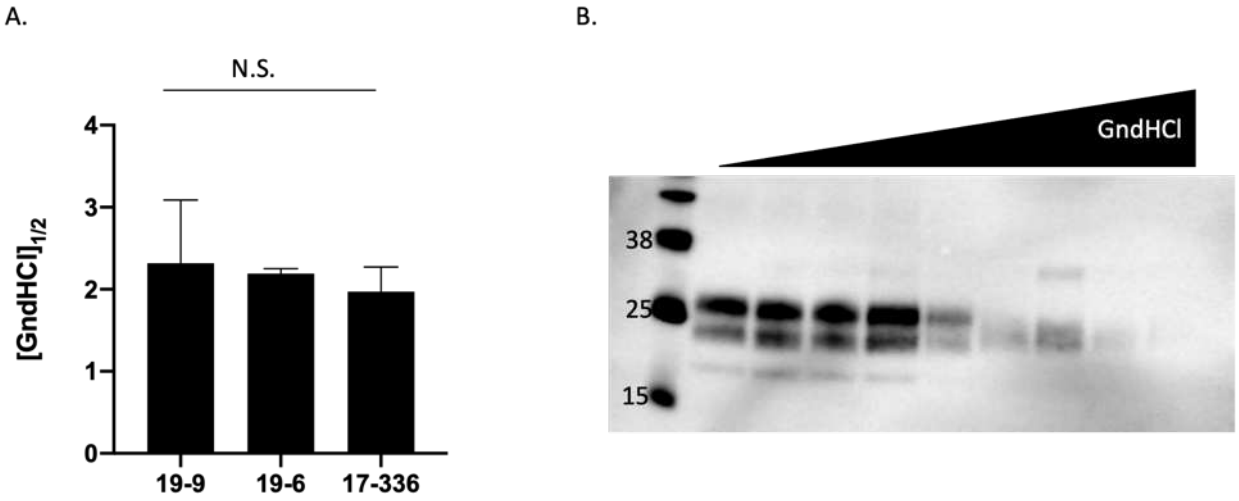

Figure 3.21. There are no differences in mean $[GndHCl]_{1/2}$ between any of the USDA isolates. There is no difference between any of the USDA isolates (A). Graph depicts mean ± standard deviation. Representative CSA blot is also shown (B). One-way ANOVA with Tukey multiple comparisons, $p>0.05$, N.S. = not significant.

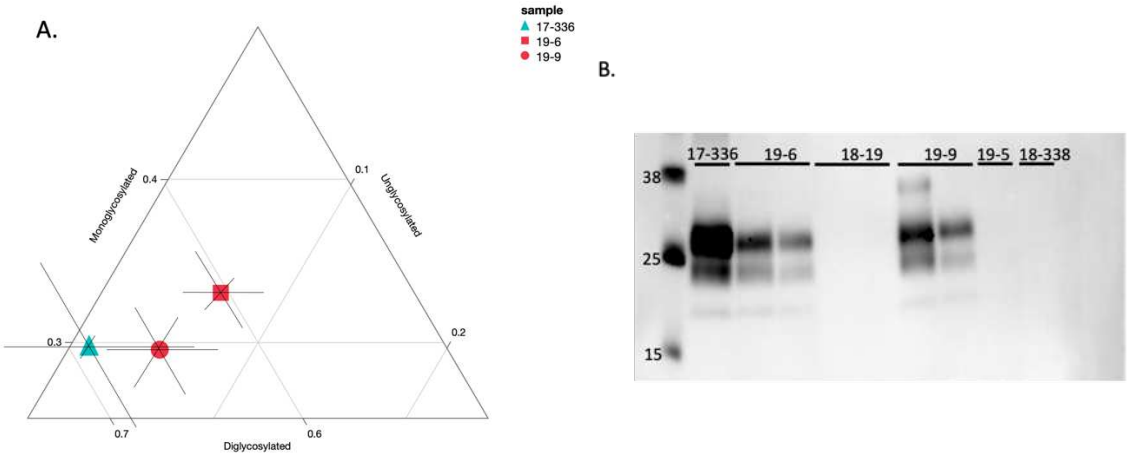

Figure 3.22. There are significant differences in glycoform ratio between USDA isolate 17-336 when compared to both 19-9 and 19-6. The significant differences between the samples was only between the unglycosylated band between 17-883 (blue) when compared to 19-6 and 19-9 (red) (A). Representative blot showing the three western blot positive samples is also shown (B). One way ANOVA with Tukey adjustment, *$p<0.05$.

Between state comparisons

To determine if there are multiple strains of CWD circulating in different states, data for isolates from a single state were all combined to generate the data for each location. Composite denaturation curves are shown in Figure 3.23. Isolates from all the different states were compared by conformational

stability based on tissue type, so only lymph node samples were compared to lymph node samples and brain samples were compared to brain samples. There are significant differences in the $[GndHCl]_{1/2}$ between Missouri and all other lymph node isolates tested and between brain samples from AR and captive white-tailed deer from the USDA (Figure 3.24).

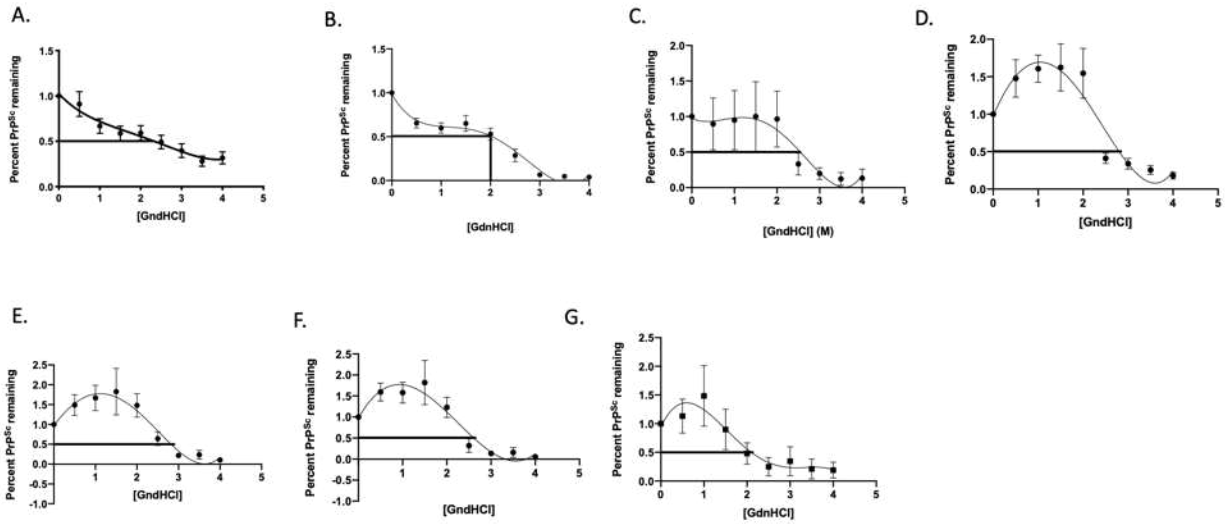

Figure 3.23. Denaturation curves for combined from each location show variability in curve shape between location. Isolates were combined for each location and fit to a fourth-order polynomial regression. Data are shown for Michigan (A, N=12), Missouri (B, N=13), Iowa (C, N=3), Virginia (D, N=9), Arkansas lymph nodes (E, N=6), Arkansas brains (F, N=5), USDA (G, N=3). Solid lines show the point at which the signal dropped to 50% of the starting signal. Data points represent the average ± SEM at each [GndHCl].

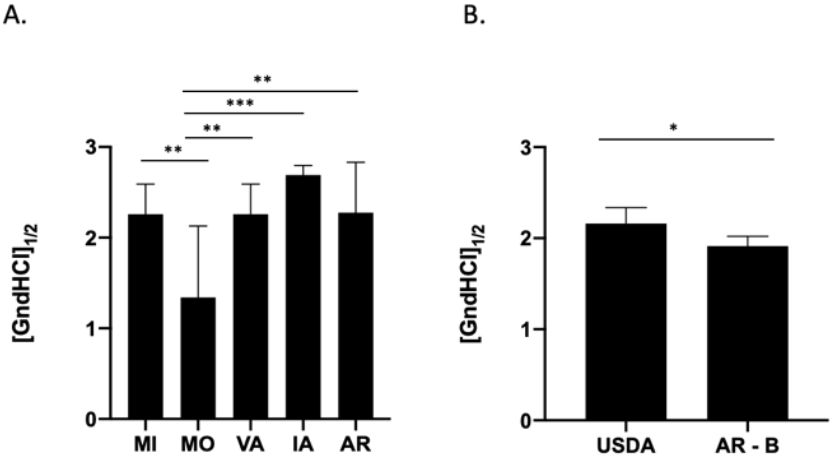

Figure 3.24. There are statistical differences in $[GndHCl]_{1/2}$ between sites. Lymph node samples that were submitted for strain typing and brain samples that were submitted were separated so comparisons were made across the same tissue type. There are significant differences between Missouri and all other states that submitted lymph node samples for strain typing analysis (A). There are significant differences between USDA captive deer samples and Arkansas samples that both

submitted brain samples for strain typing analysis (B). One way ANOVA with Tukey adjustment (A) or unpaired t-test (B). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To visualize the variability in conformational stability within sites, individual isolate denaturation curves for each location were overlayed on the same graph for each location. Generally, sites that are from lymph node-derived samples have greater variability within the samples, with quite a lot of variability noted in isolates from Michigan, Missouri, Iowa and Arkansas lymph nodes (Figure 3.25 A, B, D, and E, respectively). Interestingly, isolates from Virginia seem to cluster into three distinct groups: one group that drops below 100% of the starting signal between 0 and 0.5 M GndHCl, another group that stays at about 100% between 0 and 0.5 M GndHCl and a third group that goes above 100% of the starting signal between 0 and 0.5 M GndHCl (Figure 3.25 C). Perhaps this could indicate that there are three groupings of CWD strains circulating in Virginia with slightly different conformations influencing PK access.

While quite a bit of diversity was noted in the different isolates within each state that lymph node samples were submitted for strain typing analysis, far less variability was noted in brain samples that were submitted from Arkansas, captive deer (USDA) and individual replicates from a reference elk brain used in the lab as a control for our assays (Figure 3.25, F, G and H, respectively). Interestingly, the isolate from AR that has a signal that increases to ~3x the starting signal in the lymph node isolate is the same isolate that goes to ~300% of the starting signal in the brain sample as well (Figure 3.25, E and F, respectively). Besides the single brain isolate from AR that deviates from the other isolates from that location, the brain samples appear to have much less isolate-to-isolate variability within a location (Figure 3.25).

When comparing isolates between locations by glycoform ratio, significant differences were found between lymph node isolates from Arkansas when compared to isolates from Missouri and Michigan (Figure 3.26 A; Table 3.9). There were no significant differences found between brain-derived prions from Arkansas isolates and USDA samples (Figure 3.26 B).

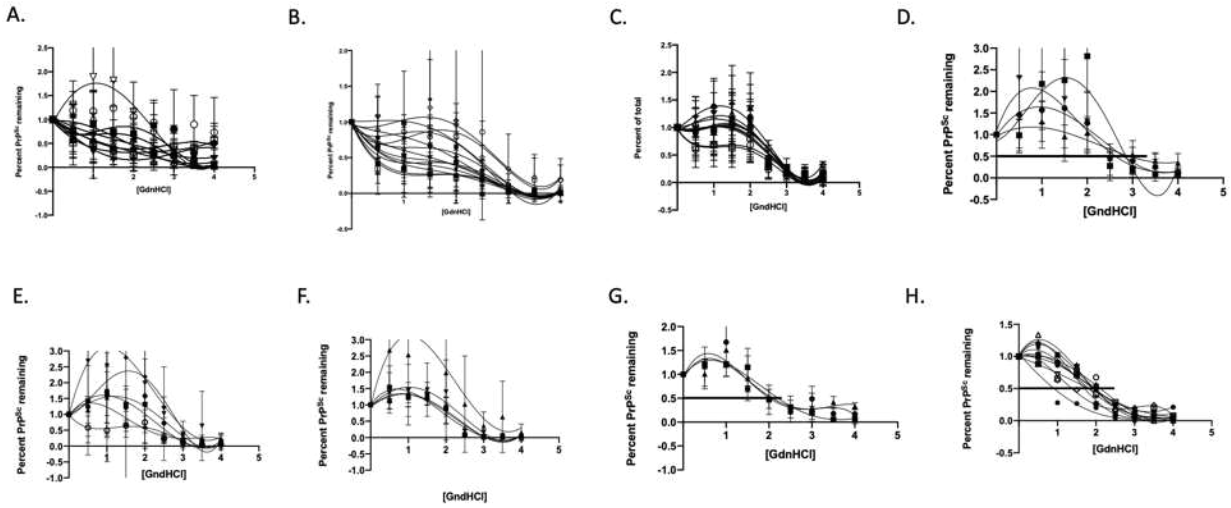

Figure 3.25. Denaturation curves for individual isolates from each location submitted for strain typing analysis showcase variability in some sites, but not others. Michigan (A), Missouri (B), Virginia (C), Iowa (D), Arkansas lymph node samples (E), Arkansas brain samples (F), USDA (G), reference sample E2 (H) are shown. Graphs show individual isolates from each location (A-G) or replicates from a reference sample (H). When performing conformational stability assay experiments, a lot of variability was noted between isolates. To visualize the variation and differences between locations, each isolate from each group was graphed individually. Graphs show mean ± SD for each [GndHCl] tested and fit to a 4th order polynomial regression.

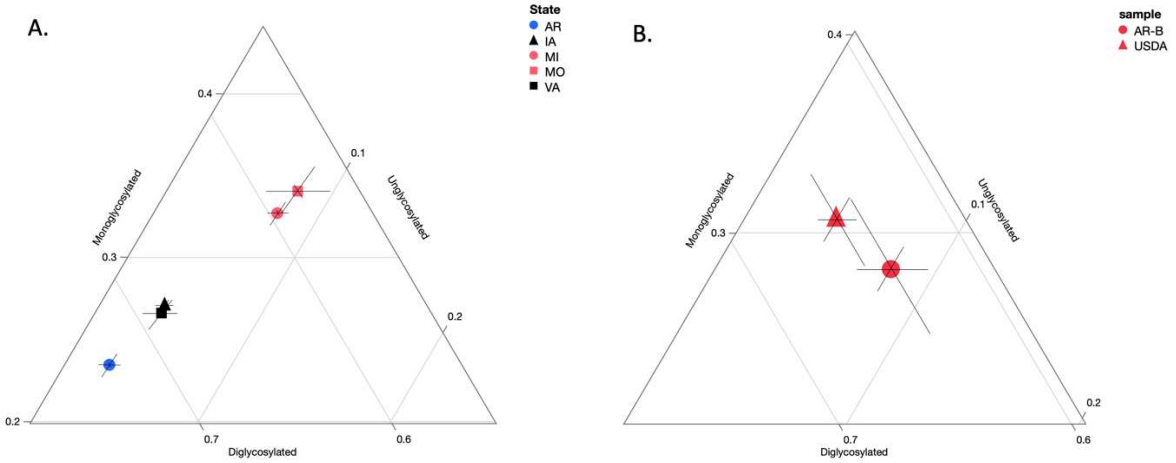

Figure 3.26. Significant differences in glycoform ratio between sites that submitted lymph node samples for strain typing analysis, but no differences in glycoform ratio between brain samples. Samples were compared within the same tissue type. Comparisons between lymph node-derived prions (A) and brain-derived prions (B) are shown. Triplots show mean ± SEM (A) or mean ± SD (B). There were significant differences found between lymph node derived prions from Arkansas (blue) when compared to Missouri and Michigan (red) (A, One-way ANOVA with Tukey multiple comparisons; differences are summarized in Table 3.9). No significant differences were found between brain-derived prions from free-ranging white tailed deer from Arkansas when compared to the captive white-tailed deer from the USDA (B, unpaired t-test, p>0.05).

Table 3.9. Summary of significant differences in glycoform ratio between AR & MI or MO lymph nodes.

Sample Comparison	Diglycosylated		Monoglycosylated		Unglycosylated	
	Adjusted p value	Summary	Adjusted p value	Summary	Adjusted p value	Summary
AR LN vs MO	0.0067	**	0.0282	*	0.0147	*
AR LN vs MI	0.0334	*	0.1545	ns	0.0573	ns

ns = not significant, * p<0.05, ** p<0.01.

Because some differences were noted between brain and lymph node prion isolates from paired animals from Arkansas (Chapter 2), all comparisons between locations were kept consistent based on the tissue from which the prions were isolated (i.e., brain or lymph node). To see if groups clustered based on tissue type, a PCA was performed using all biochemical strain typing information (CSA and glycoform ratio), while grouping samples based on tissue of origin and utilizing different symbols for the different locations (Figure 3.27). While there was no distinct clustering of the samples based on tissue type, the isolates derived from brain samples grouped much more tightly than the isolates derived from lymph node samples. The brain-derived prion samples were also clustered in the center of the plot and entirely encompassed by the grouping of the lymph node-derived prion samples (Figure 3.27).

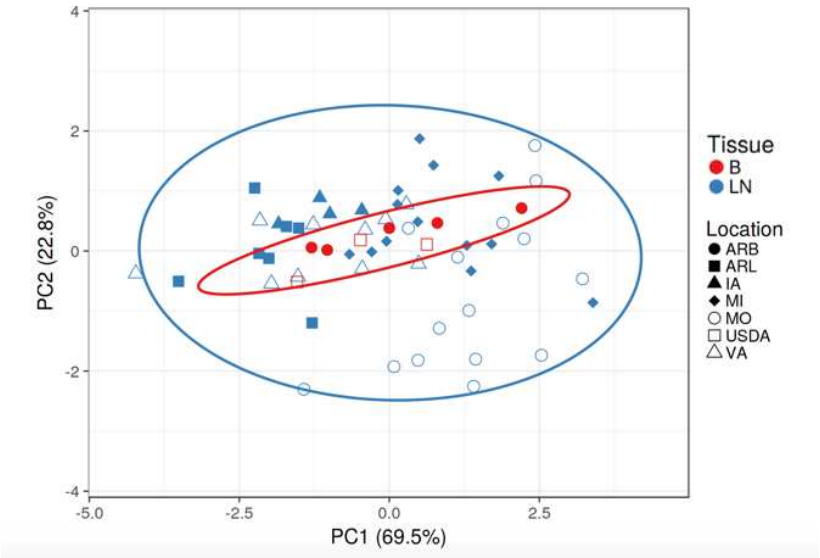

Figure 3.27. Principle component analysis of CWD isolates clustered by tissue of origin. To determine if there was any clustering or separation of samples by tissue type, a PCA was run. Location and tissue type were included as annotations and glycoform ratio and conformational stability assay were used to calculate the PCA. X and Y axis show principal component 1 and principal component 2 that explain 69.5% and 22.8% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse[27].

Finally, to determine if samples would cluster by location, another PCA was performed (Figure 3.28). Samples were separated by tissue type and compared by location between lymph node samples (Figure 3.28 A), brain samples (Figure 3.28 B) or by location regardless of tissue type (Figure 3.28 C). When looking within the lymph node samples, the isolates from Missouri overlap very little with isolates from Iowa and Arkansas, suggesting that there might be some collective differences between Missouri and the other two locations (Figure 3.28 A). Generally, the Missouri isolates do appear to separate from the other locations, suggesting CWD is different in Missouri than the other states tested. When samples were compared regardless of tissue type of origin, however, Virginia and USDA samples barely overlap, suggesting that they might have distinct clusters of CWD as determined by PCA (Figure 3.28). While there are some locations that don't appear to overlap much, there is, generally, a lot of overlap and similarity between the other locations. This suggests that while there may be some biochemical strain differences between the separated locations, CWD overall appears to have similar biochemical characteristics regardless of where it comes from. PCA on the brain isolates also show a lot of overlap and an increase in sample size would be informative to see if this trend holds true (Figure 3.28 B).

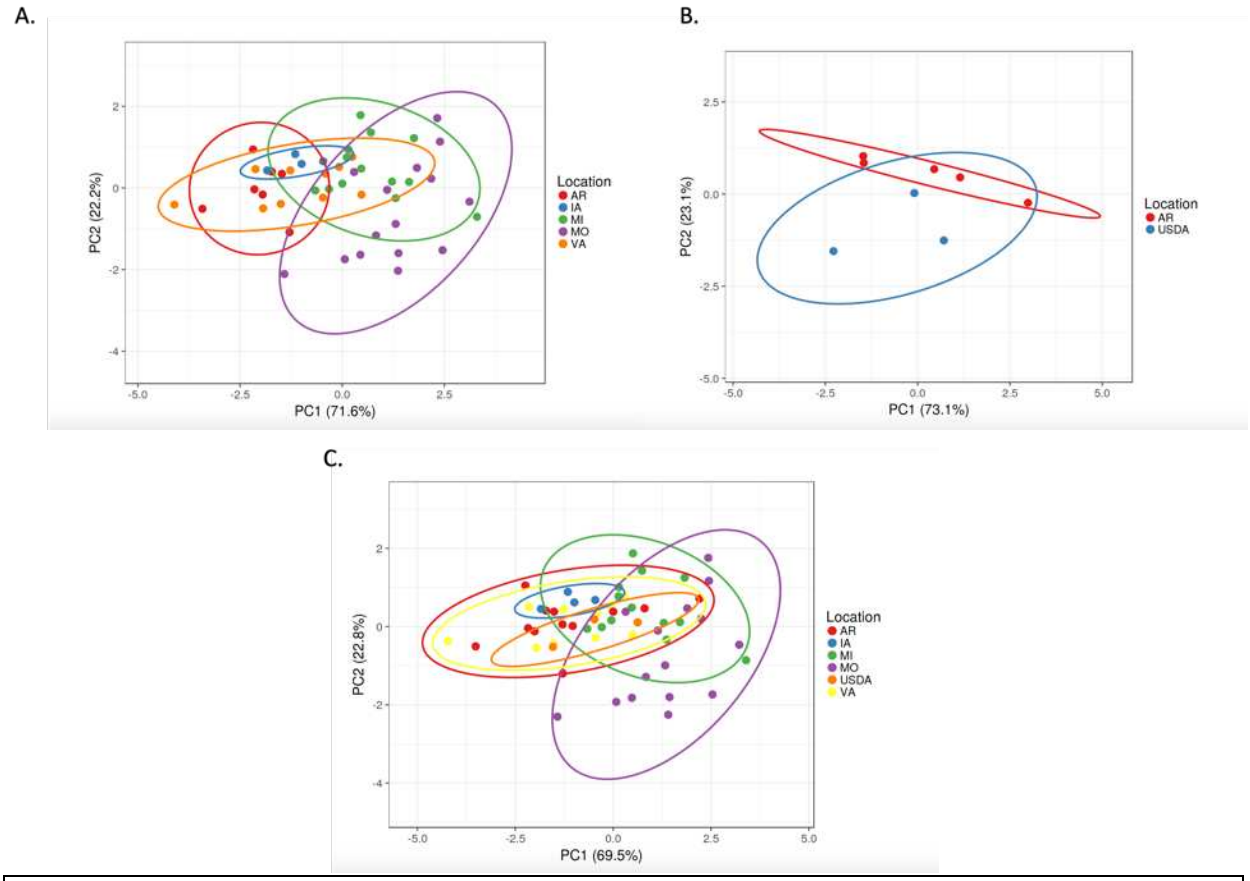

Figure 3.28. Principle component analysis of between site biochemical strain typing results. Isolates were either separated by either lymph node (A) or brain (B) or both tissues were combined (C). Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. There is substantial overlap between locations regardless of tissue type[27].

Discussion

The purpose of this work was to determine if there were any biochemical strain differences in chronic wasting disease isolates within or between locations across the United States. While a lot of work has been done to characterize CWD prions, nearly all of the research has heavily relied on mouse or bank vole bioassay and characterization of the prion only after it has been passaged through a mouse model[5,11–13]. While these studies and data are extremely powerful and have significantly advanced our knowledge and understanding of CWD, very few studies have focused on characterizing CWD prions from the animal before passage into a model. While convenient, the heavy reliance on mouse models may result in a purification step of prion strains, select for specific CWD strains or influence the emergence of strains that wouldn't be replicated between deer in a natural setting. Additionally,

understanding the diversity and distribution of CWD prions could provide some insight into the origin of CWD and the evolution over time. For these reasons, this project was started to assess possible biochemical prion strain differences from naturally infected cervids. Isolates from Michigan, Missouri, Arkansas, Iowa, Virginia and captive animals from three deer farms from two distinct regions were submitted for strain typing analysis and conformational stability, glycoform ratio and electrophoretic mobility were assessed. Significantly, there are statistical differences between some of the sites tested and between captive and free ranging deer solely utilizing biochemical techniques. These results are the first step to understanding the diversity and distribution of CWD prions.

Michigan

Michigan found its first case of CWD in a captive deer farm in Kent county in 2008 and the next positive case was in a free-ranging white-tailed deer in lower Michigan in 2015. Since then, CWD has been found in multiple counties (Figure 3.1, 3.2). More cases of CWD were found in captive deer in 2017 in two captive deer in Mecosta county. CWD was first discovered in a four-year-old doe in Upper Michigan in 2018 in Dickenson county near the border of Michigan and Wisconsin (which has had CWD since 2002). The origin of the first cases of the free-ranging white-tailed deer in Michigan is unknown[29].

For this study, Michigan submitted 13 samples for strain typing analysis and provided information as to the animals' genotype, haplotype, age, sex and within-state distribution (Table 3.1). When assessing these isolates by conformational stability, three samples had to be excluded from the analysis because the signal never dropped below 50% of the starting signal or because there were two few replicates to include to get an average value (Figure 3.3 J, K and L, respectively). While there was no difference in mean $[GndHCl]_{1/2}$, three general shapes of the denaturation curves were observed when looking at the shape of the denaturation curves. Some of the isolates appeared to have a classical CWD denaturation curve, where the signal decreases below the starting value, but doesn't drop below 50% immediately or rises above the starting signal and then drops below 50% of the starting signal (Figure 3.3, A, B, D and H)[24]. Other isolates had denaturation curves where the signal quickly and steeply dropped below 50% of the original starting signal (Figure 3.3, F, I), and other isolates "smiled" where the signal quickly dropped below 50% of the starting signal and then again had signal that exceeded (or nearly exceeded) 50% of the starting signal (Figure 3.3 C, E, G).

Samples that have a more typical denaturation curve may be classical CWD, whereas the samples that are less stable and drop below 50% of the starting signal represent a different, less stable strain of CWD. Importantly, less stable prion isolates have been shown to be the most neuroinvasive. These isolates may have prions that are more likely to transmit between deer by direct contact and rapidly cause neurological disease. To determine if this is true for these isolates, bioassay should be employed. If true, this has significant implications for predicting clinical disease and CWD spread in these locations.

For the isolates that "smile", there may be multiple quasi-species in these animals with one prion species that is less stable in the presence of GndHCl and another, less abundant, that is more stable. This more stable strain may be represented in the increased signal/the lack of the signal being extinguished in these samples. It is also possible that the other prion quasi-species are folded in such a way that the epitopes are not exposed until they are exposed to higher concentrations of GndHCl that then expose the epitope, resulting in the increase in signal at these high concentrations. It would be interesting to passage the "smiling" isolates through bioassay in transgenic mice to see if two unique prion strains are able to be isolated (a situation similar to hyper and drowsy[30]). Further studies should examine additional tissues from the same location a few years after these samples were collected to see if there was any strain stabilization over time. Maybe one of the strains is more transmissible than the other and there would be a singular prion strain detected or there could be two distinct strains circulating, one being very stable and one being very unstable. Perhaps only the more stable strains are able to persist in the environment and transmit to other animals more easily through an indirect route and only the high-stability strains would be selected for. Conversely, less stable prion strains have been shown to be more neuroinvasive and if the less stable strain is selected for and transmitted perhaps we will see a rise in CWD cases[31]. These samples suggest that there are more quasi-species in these animals. Examination of isolates from these same locations at a later time point will be extremely interesting to observe strain emergence and evolution over time. While the shape of the curves did appear to be different, there was no difference in the average $[GndHCl]_{1/2}$ between any of the isolates (Figure 3.4).

While running the samples by Western blot to analyze by glycoform ratio, it was noted that there was often a unique "doublet" in the signal of the diglycosylated band (Figure 3.6 A). Interestingly, this

doublet was observed with regularity when western blots ran well and there was a good band separation. This electrophoretic pattern had not been observed before in any of the prion blots run in the lab. To determine if this electrophoretic pattern was the result of an alternative PK digestion site, isolates were treated with PNGase F as described in the methods. If there were differences in PK digestion sites, two bands would be expected after PNGase F and PK treatment, but only one band is observed (Figure 3.6 C). These results indicate that the doublet is not a result of an alternative PK digestion site. These findings could be explained by alternative glycan trees, which could be tested by running these isolates on a 2D gel or utilizing PNGase F again to isolate the glycans and assess their composition using mass spectrometry.

Alternatively, it is possible that there is a protein running at the same size that is interfering with the detection of the prion protein. In fact, proteinase K, which is used in every one of our assays to discriminate between PrP^{C} and PrP^{Sc}, is 28.9 kDa in size and runs at nearly the exact molecular weight of digested, diglycosylated PrP^{Sc}. While this is a likely explanation for the data shown here, it is interesting that this "doublet" has only ever been observed in free-ranging animals and has not been noticed in any of the captive isolates examined (representative captive image in Figure 3.21). PK interference is likely the culprit here, but future work assessing the glycan trees would be informative as to if there are any differences in the glycosylation of these free-ranging animals and if this biochemical difference has any biological implications.

Finally, to assess in there was any clustering or grouping of samples from either location 1 or 2 in MI based on biochemical strain typing results, age and haplotype, a PCA was constructed (Figure 3.7). Genotype was not included because there was only 1 isolate with a different genotype and that information would not be very informative in the construction of the PCA. While there was substantial overlap between all samples from Michigan, including those from both locations, isolate 506421 wasn't clustered as closely with the other isolates from either location. Interestingly, 506421 was the only isolate from location 2 that gave interpretable conformational stability data and had glycoform ratio data that was significantly different from 5 other Michigan isolates. While all the isolates had primarily diglycosylated PrP^{Sc} similar to classical CWD, isolate 506421 had di- and monoglycosylated PrP^{Sc} contributing a more equal amount to the total signal more similar to the E2 elk isolates used in the lab (data not shown).

These glycoform ratio data and PCA visualization suggest that isolate 506421 might have some unique strain properties that are more similar to elk CWD. From location 2 overall, the signal from samples 421858 and 455702 were quite weak and only 421858 was able to give interpretable data for glycoform ratio, although it was a low signal, and neither of those samples gave interpretable results for conformational stability. The only other member of group 2, 507416, did not give interpretable data by conformational stability assay because the signal never dropped below 50% of the starting signal. While a $[GndHCl]_{1/2}$ wasn't calculatable, 507416 did have strong signal by glycoform ratio. Together, this data demonstrates that there is a lot of diversity in CWD strains in location 2.

Perhaps some CWD circulating in and around location 2 from this study is more PK sensitive and/or much less sable than CWD from location 1 (i.e., 421858, 455702). This would be interesting and could indicate that there are two distinct clustering of CWD in Michigan that may progress slightly differently in these different populations. These results could also be explained by these animals being much earlier in the disease course and simply not having enough PK-resistant PrP^{Sc} to be detected by our relatively low-sensitive western blot assays, but this is not expected since all animals were confirmed CWD positive prior to submission. On the other hand, isolate 507416 might be an exceptionally stable prion strain and these data are highlighting how variable CWD is in location 2. Overall, there appears to be something interesting happening in location 2 in Michigan that isn't clustering with other Michigan isolates. Assessing additional isolates using biochemical strain typing techniques at a later time to see if these strain features are maintained in populations over time would be informative to strain maintenance, stabilization, evolution and emergence.

Another isolate from Michigan that is worth discussing is 508792. This isolate is particularly interesting because it is 96SS, which is the genotype of white-tailed deer associated with resistance to CWD[32]. Furthermore, this animal is diplotype CC and Brandt *et al.* found haplotype C to be associated with reduced risk of CWD[28]. Together, these genetic results suggest that this animal would likely be more resistant to disease and have a longer disease course[33–37], but this animal was only 1 year old at the time it was tested positive for CWD. Perhaps this is an example of vertical transmission where this animal was exposed *in utero* and this can explain how this genetically resistant animal was testing positive for CWD at a young age [20,38–40]. While these genetic findings suggest that a different strain may emerge, this

isolate was, biochemically, quite similar to the rest of the isolates from location 1. Further work utilizing bioassay of both 96GG and 96SS would be informative to understand the transmission dynamics of this strain[41].

Overall, the results from Michigan indicate that there is something unique about the isolates from location 2 and there is evidence of quasi-species within some animals. Additionally, isolate 506421 was statistically different from a number of isolates in glycoform ratio and didn't cluster tightly with many of the other isolates from Michigan, suggesting this isolate may also represent a unique prion strain. Future work analyzing additional isolates from these locations will provide important insight as to if these strain properties were maintained over time. Additional bioassay work to assess if these biochemical differences/findings translate to a difference in biological outcome will also be an important next step in this work. Importantly, these studies highlight the variability in Michigan and imply there are strain evolution events occurring in these CWD clusters.

Missouri

Chronic wasting disease was first discovered in Missouri in a captive deer in 2010 and in a free-ranging white tailed deer in 2011[42]. While CWD is present in the state, it is still relatively rare. Missouri submitted a total of 16 samples that were included for biochemical strain typing analysis. Samples clustered into 7 distinct outbreaks. For the purpose of analysis, isolates were then grouped into three main groups to have increased numbers of isolates from each group. Genotyping for these samples was performed by me. All animals that had amplifiable DNA that could be submitted for genotyping analysis were 96GG (Table 3.3).

Samples were assessed for conformational stability and as was observed with the Michigan isolates, different denaturation curve shapes were observed between Missouri isolates. For about half of the samples, the signal quickly drops below 50% of the starting signal around treatment with ~1 M GndHCl, implying that these isolates are unstable in the presence of GndHCl (Figure 3.9 C, D, G, I, J, K). The denaturation curve for the rest of the isolates behave typical of a classical CWD isolates – the signal gradually decreases until it loses 50% of the starting signal at a higher concentration of GndHCl (Figure 3.9 A, B, E, F, H, L, M). Unlike the Michigan samples, the differences in the shape of the denaturation curve will be represented in the $[GndHCl]_{1/2}$ more accurately because none of the samples cross the 50%

line more than one time and the difference in the curve is directly related to the calculation of the $[GndHCl]_{1/2}$. The different shapes of these curves imply two groups of structurally different CWD strains circulating in MO.

Missouri isolates were compared to see if there were any significant differences in the average $[GndHCl]_{1/2}$ between any of the isolates. While some of the isolates, most notably 20026466, have a much lower $GndHCl_{1/2}$ than some of the other samples there were no significant differences between any of the samples (Figure 3.10, one-way ANOVA with Tukey's multiple comparison, $p>0.05$). Even when using a Dunnett's test to compare 20026466 to the other Missouri isolates, there were no significant differences ($p>0.05$). The low sample size is likely contributing to the lack of significant differences (there were only two successful replicates for isolate 20026466). While not statistically significant, these results indicate that this sample should be utilized in further analyses to assess additional strain characteristics.

When comparing samples by glycoform ratio, there were many significant differences between the Missouri isolates (Table 3.4). Without focusing too much on any single significant comparison, the takeaway from these multiple comparisons is that there are more differences between the isolates in Missouri than the isolates in Michigan. This could mean that there is greater variability in the CWD circulating in Missouri deer. While some of the isolates have di- and monoglycosylated bands that contribute a more equal amount to the total signal, the diglycosylated form contributes to the majority of the signal as would be expected with a classical CWD prion. While running blots for glycoform ratio analysis, a doublet in the diglycosylated band was again noted frequently in the Missouri isolates similar to the observations made from the Michigan isolates. Because of the findings with the Michigan isolates and the number of samples we received for biochemical strain typing, it was decided to not pursue the nature of this doublet because it was likely sample interference from the proteinase K. The isolates provided from MO were older and more decomposed than others and, while the prion signal is retained, perhaps the glycan trees broke down and gave more equivocal results, resulting in increased statistical differences between the Missouri isolates.

To determine if the isolates from Missouri clustered by within state distribution, samples were plotted using a PCA. When samples were separated by all 7 internal locations, there was no clear, distinct clustering between the sites, but site 5 had a tighter cluster of the isolates than the other

locations, suggesting the isolates from this location were more similar (Figure 3.12 A). When sites were combined for the sake of larger sample sizes in each location and projected on a PCA, it was noted that the isolates from location 1 were more spread out than the isolates from location 2 and location 3 (Figure 3.12 B). Interestingly, location 1 represents the area within Missouri that has had CWD the longest (J. Batten, personal communication)[42]. One of our hypotheses was that locations that had CWD the longest would promote the emergence/selection of a more homogeneous prion strain; however, this is not what we observed. Perhaps the longer CWD is circulating in a population and in the environment, biochemically different strains are able to emerge as they are passaged between animals and the environment[43]. To test this hypothesis and determine if this phenomenon is observed in other populations, we will look at CWD strains from Colorado (the original site of CWD) and compare the results of new and archived CWD strains from CO and elsewhere.

Overall, the isolates from Missouri displayed a lot of variability in the biochemical characteristics in conformational stability (although none of the differences were statistically difference) and a lot of statistical differences were found in glycoform ratio. Most interestingly, the greatest variability with the widest spread of the data was from the location that had CWD the longest, which was not what we hypothesized. Ongoing bioassay experiments will help determine if the biochemical differences/variability observed relates to any biological differences in disease outcome in transgenic mice. Most interesting, but also most difficult, would be to note if there are any differences in disease presentation in the white-tailed deer from Missouri. Missouri is a location to continue monitoring for emergence of novel CWD strains and future experiments assessing biochemical characteristics from natural isolates will be informative to assess the emergence and evolution of prion strains.

Arkansas

Chronic wasting disease was first discovered in Arkansas in 2016 from an elk from the 2015 hunting season, but later testing indicated that CWD was likely in the state for decades. It is thought that the transport of a potentially infected elk from Colorado/Nebraska to Arkansas was the first introduction into the state in 1981-1985. With that in mind, it is surprising that there was not a case discovered earlier since CWD surveillance has been going on since 1998, but perhaps infections were missed simply by chance until 2015. Another sick, free ranging white-tailed deer was observed in 2016 and an initial

sampling around the location of the infected animal found a 23% positivity rate in surrounding herds. Since the first discovered case of CWD in Arkansas, the disease has spread across 14 counties. Arkansas is actively trying to control CWD[44].

While differences within site by biochemical analysis was shown and discussed in Chapter 2, it is relevant to mention here that the data collected, analyzed and shown in chapter 2 was included and incorporated into the between site comparisons. Interestingly, when looking at differences in conformational stability and in glycoform ratio across the lymph node isolates, there were no differences in conformational stability and the only differences in glycoform ratio were between a sample that had low signal on western blot, which may have contributed to some of the statistical differences observed. Altogether, these data suggest that the strain of CWD circulating in Arkansas, at least from the isolates examined in this study, is typical of classical CWD. Because the origin of CWD in AR is so well understood, we hypothesized that AR CWD would be similar to classical CWD, which does appear to be the case. Based on these findings, our hypothesis is supported that Arkansas CWD is classical CWD, but the variability observed in the lymph node isolates doesn't exclude the possibility of novel strain emergence in AR (Chapter 2).

Virginia

In 2009, Virginia detected its first case of CWD in a free-ranging white tailed deer along the West Virginia border (WV discovered its first case of chronic wasting disease in 2005)[45]. Since the initial case, CWD spread across the West Virginia/Virginia border until 2018 where the first case was found South East of the initial case is Culpepper county for the first time[46].

For the purposes of this study, a total of 14 samples were submitted for biochemical strain typing analysis: 11 positive samples and 3 negative animals. Our assays revealed that we had interpretable data from 9 of the samples and 5 of the animals did not have interpretable results by our assays (Table 3.5). To date, we have not yet been able to get in touch with our collaborators in Virginia, so it has been assumed that our data are congruent. For at least 2 of the samples that we found to be negative the animals had tested positive, suggesting that these are also either a PK-sensitive strain of CWD or the animals did not have enough PrP^{Sc} to detect using our assays. When assessing the samples by conformational stability, the samples had remarkably similar denaturation curves and there were no

statistical differences between the mean $[GndHCl]_{1/2}$ between any of the samples from Virginia, but when denaturation curves were overlayed (Figure 3.25 C), there were subtle differences in the denaturation curves that clearly showed three distinct denaturation curves. These three different groups, coupled with several differences in glycoform ratio between many of the isolates suggests that there is diversity in the isolates from VA. While many of the findings were reminiscent of classical CWD, the biochemical differences and grouping of samples by denaturation curves imply some strain evolution is occurring. Following up with additional samples from later time points will be informative as to the evolution or stabilization of these strains over time.

Iowa

Iowa detected its first case of CWD in 2012 in a white-tailed deer hunted on a hunting preserve[47]. The animal was found to have come from a deer farm in the central part of the state, that ultimately ended up with an 80% CWD positivity rate when the animals were ultimately culled[48]. Since then, cases have been found in 8 counties, with all but one bordering Wisconsin, Illinois and Missouri, where CWD has was found prior to its discovery in Iowa. The other county where CWD has been found borders Nebraska[47].

From Iowa, 8 lymph node samples and 1 skeletal muscle samples (070) were submitted for strain typing analysis, but only 4 lymph node isolates had detectable signal by our assays to utilize our biochemical strain typing assays (Table 3.7). Of the four samples that we were able to successfully get data from, they had remarkably similar denaturation curves and average $[GndHCl]_{1/2}$ values. There was only one statistical difference in the unglycosylated band between isolate 191058 and 197186. While a subtle difference, it is interesting that isolate 191058 is from a geographically distinct location than the rest of the isolates (R. Ruden, personal communication). Perhaps there are differences in CWD that will evolve to be more diverse over time. Overall, CWD circulating in Iowa appears to be a classical CWD strain that hasn't evolved since its emergence/introduction. Of the other samples that were submitted for strain typing analysis that didn't give data in our biochemical assays, there are three possibilities: 1) these samples were just too early in the disease course to have sufficient levels of PrP^{Sc} to analyze using our biochemical strain typing techniques, 2) these isolates could represent a PK-sensitive prion strain that is unable to be detected by our assays but may be significantly contributing CWD transmission, or 3) these

isolates could be in a unique conformation that does allow access by the antibody used. Further experiments with additional antibodies would answer these questions.

One isolate interest is C603, which gave interesting results on conformational stability and glycoform ratio analysis (data not shown). This sample appeared to migrate and look exactly like PrP^C. This result was so surprising that it was initially assumed that something had gone wrong with the experiment and PK had not been added to the tubes. The data were, however, repeatable over four separate blots. This could represent a unique prion strain that is PK resistant and doesn't migrate as we would expect for a prion or is conformationally unique such that the PK-sensitive portion of the protein is no accessible in this isolate. Additional work looking at seeding ability, conformation and infectivity of this sample would be informative.

Overall, the results from Iowa indicate that the CWD that was detectable by our assays is pretty similar to classical CWD and there is a small difference between two of the isolates from geographically distinct locations. Further work looking at the locations from the isolates with statistically different unglycosylated PrP will be interesting to determine if distinct strains evolve from these parent strains in these locations. The samples that didn't give interpretable data may represent a PK-sensitive CWD strain and there were some interesting findings in some of the isolates that would be interesting to follow up on using additional techniques to look at seeding activity or infectivity using PMCA and/or bioassay. Perhaps these PK-sensitive prions are a novel strain or have distinct characteristics compared to the other isolates examined.

USDA

Isolates from the USDA were from farmed animals unlike the isolates from the various states that were all from free-ranging animals. This was exciting because we were hoping to be able to compare prion strains from free-ranging and captive deer. Unfortunately, the only tissues we were able to procure from the USDA were brain samples, excluding the obex, so we were limited in the number of locations we were able to compare to. We hypothesized that isolates from farmed deer would be more homogenous because all the animals would be exposed to a single introduction event and the prions would become more similar as they infected and passaged from animals in a closed environment.

From the USDA, all the samples that were received were brain samples from white-tailed deer. Of the 19 samples that were received for strain typing analysis, only three isolates gave interpretable data by our biochemical strain trying assays. Because we received brain samples that did not include obex, it is possible that the samples that didn't give us positive signal by our methods because the sample didn't have prions that had migrated through the rest of the brain past the obex[22]. To ensure that there weren't any anomalies in protein concentration, a BCA was also run to ensure that protein concentration was as expected and samples all had an acceptable protein concentration. Because we expected most of the samples to be CWD positive, RT-QuIC was performed to test the samples for seeding activity. While the results presented here can be explained by low amounts of PK-resistant PrP^{Sc} in the brain, it is also possible that some of these prion strains are PK-sensitive because they have high seeding activity but no PK-resistant PrP^{Sc}. Isolate 18-19, for example, was able to seed an RT-QuIC reaction at a rate that equaled or exceeded the samples that came up positive by western blot that also gave positive results by RT-QuIC (Figure 3.19). While PK-resistant PrP^{Sc}, infectivity, and seeding reactions are not directly related, these results imply that this strain is PK-sensitive or is early in disease course such that PK resistant plaques have not yet formed – in fact, oligomers form before PK resistant plaques have been shown to be more infectious and PK sensitive as compared to plaques[49,50]. In short, while many of these samples were unable to give us positive results in our biochemical strain typing assays, these samples are interesting in that there may be too low of a titer of PrP^{Sc} plaques to detect in our assays, PK sensitive, or both.

Of the three samples that were able to give us conformational stability data, the denaturation curves and average $[GndHCl]_{1/2}$ were similar and there were no statistical differences between any of the samples and was similar to classical CWD (Figure 3.20, Figure 3.21). When comparing samples by glycoform ratio, isolate 17-883 was statistically different from 19-9 and 19-6 in the unglycosylated band (Figure 3.22). These results are interesting because isolate 17-883 is from one deer farm located in the southern US, whereas samples 19-9 and 19-6 are both from deer farms in the eastern US (Table 3.8). These findings are interesting because the only significant differences between isolates in Iowa are in the unglycosylated band between two isolates from geographically distinct farms. These data indicate that CWD from farmed white-tailed deer is very similar, especially in stability, but there are differences in the

unglycosylated band between the two states that had isolates that were usable by these biochemical assessments. Interestingly, this follows the same trend that was observed in Iowa, where the only difference between geographically distinct isolates was observed in the unglycosylated band. These findings may indicate evolution occurring within different deer farms changing the emergence of different strains.

Between state comparison

In addition to comparing isolates within a location, another main goal of this research project was to determine if there were any differences between states. For the purposes of these comparisons, all samples from a state were combined to generate values for that location despite any within state location differences or biochemical differences that were noted. Interestingly, we found that CWD from Missouri had a significantly lower average $[GndHCl]_{1/2}$ when compared to all other locations that submitted lymph node samples for strain typing analysis (Figure 3.24). This data suggests that there is something unique about the stability of the CWD isolates from Missouri. Because Missouri isolates overall have a lower conformational stability, it would be interesting to assess how environmentally stable these prions are. The low conformational stability would suggest that these prions would have less longevity in the environment and perhaps management techniques in Missouri will be effective in a shorter amount of time if cervids are prevented from entering contaminated environments. These data also suggest that while CWD is generally a very stable prion, some CWD strains may emerge that are less stable. If these strains are indeed less stable in the environment, this would be great news in the control of CWD in cervid populations. Future experiments investigating this finding on the environmental stability of CWD isolates from Missouri would be informative. Conversely, as mentioned above, less stable prion strains are thought to be the most neuroinvasive. Perhaps these strains are less environmentally stable but perhaps if transmitted vertically or with direct contact will cause clinical disease more rapidly.

Because our results from chapter 2 comparing brain-derived and lymph node-derived prions suggested that there may be some differences based on tissue type, the captive deer from the USDA were not compared to the other states that submitted lymph node samples. Instead, brain isolates from Arkansas and isolates from captive white-tailed deer from the USDA were compared by conformational stability (Figure 3.24). There was a significant difference between captive deer and free-ranging deer from

Arkansas. These results are interesting because they suggest that there may be differences in prion strains between captive and free-ranging deer. Perhaps strains are evolving differently in free-ranging deer because there are more diverse environmental factors that are influencing strain properties or transmission between multiple cervid species (elk and deer, perhaps). Increasing the sample size would be interesting to see if this observation is maintained.

When trying to look at the conformational stability in each site, individual isolate denaturation curves were overlayed from the different sites, as well as a reference elk brain samples (E2) frequently used in our lab as a control CWD isolate. When these graphs were made, it was noted that the lymph-node derived isolates had quite a bit of variability that was condensed when combined into a singular denaturation curve for the site, whereas the brain samples seemed to have much less variability between the denaturation curves for the individual isolates (Figure 3.25). E2 was shown to demonstrate how tightly replicates from a farmed elk and, more importantly, a single brain isolate, group together. The variability of the lymph node samples may be because there is a greater number of quasi-species within the lymph node of infected animals contributing to the variability within an animal or lymph node samples being more diverse in biochemical characteristics. While strain typing wasn't done in this study, research has shown that extraneural prions have more zoonotic potential[51], perhaps because of greater strain diversity in these peripheral tissues as represented by the differences between (and within) these isolates. Interestingly, when brain and lymph node tissues were visualized by PCA, the brain-derived prions were clustered in the middle of the lymph node isolates, demonstrating the increased variability seen within the lymph nodes as compared to the brain (Figure 3.27). As noted, this analysis demonstrated that there is generally more variability in the lymph node samples than in the brain isolates. While this may have a biological implication and is nonetheless interesting, it is also possible that the technical difficulties of working with lymph node tissue as opposed to the ease of working with brain samples contributed to this finding.

It is also interesting to note how tightly the denaturation curves group for the isolates from farmed deer from the USDA as compared to the lymph node samples (Figure 3.25). While a number of CWD outbreaks in free-ranging deer can be traced back to (or radiate from) captive cervids, it is possible that infected deer are depositing prions into the environment that are then passaged regularity through the

other deer on the farm, resulting in purifying selection of a prion strain within the captive herd that would permit for more strain adaptation once circulating through free-ranging animals, especially because multiple cervid species can overlap in environments. While these results are interesting and suggest that perhaps there is a more homogenous prion strain in captive deer (from a particular deer farm as there were some glycoform ratio differences between farms), but the sample sized in this study are quite small. Research incorporating additional farmed deer would be informative to address this question.

Glycoform ratio differences were also found between Missouri and Arkansas, as well as Arkansas and Michigan. It was surprising to see differences between Missouri and Arkansas because they share a border and, presumably, deer would cross from one state into another and/or hunters may transport deer carcasses or contaminated equipment across state lines. While there were some significant differences, all locations are primarily diglycosylated, as is typical for classical CWD (Figure 3.26 A). These data provide strong evidence of biochemical strain differences between some of the states used for CWD strain typing analysis. There were no significant differences in glycosylation patterns between the USDA and brain-derived prion isolates from Arkansas (Figure 3.26 B). While not statistically different, there does appear to be more di- and monoglycosylated PrP^{SC} in USDA samples and perhaps increased sample size would result in statistical differences. As discussed above with the Michigan isolates, it is interesting to note that the diglycosylated “doublet” was only ever observed in free-ranging animals and never in any of the captive deer from the USDA (Figure 3.23). This may be the result of tissue differences, although experiments with an experimentally inoculated deer also didn’t show a doublet (data not shown). At this point it’s unclear if these differences are truly biochemical or if it is simply due to PK interference, but it was an interesting observation. Bioassay would be an interesting way to determine if there are any biological implications of this finding.

To see if there was any grouping of isolates based on tissue of origin, all brain and lymph node isolates were plotted using a PCA to see if there were any distinct groupings of the isolates by tissue type (Figure 3.27). While the brain and lymph node clusters completely overlapped, the brain derived samples from Arkansas and the USDA grouped tightly together and in the center of the lymph node cluster. This data visualization supports the hypotheses posed by some of the biochemical strain typing data, implying that brain samples are more similar to each and with far less variability than the lymph node derived

isolates. Again, this suggests that there is greater variability in the lymph node derived samples than in brain-derived samples. Future studies poised at assessing prion strains from cervids should include assessment of lymph node derived prions as these are the most likely to be passaged from individual to individual or consumed by other animals[20,26,52–56].

Finally, when samples were visualized by PCA either divided by tissue type or compared all together, a substantial amount of overlap was noted. Because there is incomplete information from all of the different locations, PCAs were only able to be constructed based on biochemical strain typing results. The addition of age, sex, genotype and haplotype information may provide interesting insights as to how isolates may cluster. With the data available, however, PCA visualization of the data suggest that there is substantial overlap in CWD biochemical characteristics, but when comparing between LN isolates, the samples from Missouri group more separately from the other locations, suggesting that there is a novel type of CWD circulating in Missouri (Figure 3.28). While there is quite a bit of overlap in PCA, this work has noted several isolates and outbreaks that should be followed up with to observe any strain evolution and emergence in the future.

As described, the overall purpose of this work was to address potential strain differences by looking at isolates from a natural host. While our results are suggestive that there are some differences between individuals (e.g., MI isolate 506421) and, notably, between Missouri CWD and other locations, these data alone cannot be used to conclude that there these isolates are different, distinct strains. Rather, my data provides evidence suggestive of novel strains. While my data suggests that there are some differences between isolates based on these assays, I do not believe that we can conclusively use only this data to call something a new strain, but we can use it to determine isolates or differences that are suggestive of a novel strain and identify isolates that warrant follow up, strain typing analysis. While biochemical differences are a critical aspect to prion disease phenotypes and even a single difference is highly suggestive of novel strains, I believe that at least one biological difference is requisite to qualify something as a novel strain, whether that is a host range expansion/contraction, incubation period, disease presentation or another biological difference.

In conclusion, this work highlights a number of interesting biochemical differences within and between sites, highlights the diversity of CWD circulating across the US, and suggests that there is

evolution of CWD occurring in free-ranging and captive populations. While the purpose of this study was to understand and characterize CWD prions before passage through a mouse model, bioassay would be an important next step to understand any biological implications of these biochemical differences. Further research to expand on this work examining new isolates from the same locations would be interesting to see if there any of these findings are maintained over time. CWD researchers should continue to characterize prions from the natural host to better understand the variability of the infectious agent in the natural host.

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Chapter 4:

Novel biochemical presentation of a CWD isolate from a captive white-tailed deer from Texas, USA, was infectious to cervidized mice and showed no evidence of zoonotic potential

Summary

Numerous studies have shown that there are multiple strains of chronic wasting disease infecting cervid species. The devastating effects of CWD on cervid populations and a recent case study suggesting an instance of zoonotic transmission of CWD to a human necessitate continued investigation into CWD strains. In an effort to participate in our strain typing study, two white-tailed deer isolates from Texas were submitted for strain typing analysis; however, the novel biochemical findings required that these samples get a more thorough investigation and their own separate report. Both isolates, TX1 and TX9, had unique biochemical characteristics but only TX1 had seeding ability in RT-QuIC and was infectious to transgenic mice. Neither TX1 nor TX9 had any evidence of zoonotic potential. The work presented here highlights the importance of continued characterization of CWD isolates/strains and demonstrates that CWD prions can be infectious even when they present in an atypical biochemical fashion.

Introduction

Chronic wasting disease was first observed in captive mule deer the late 1960's and reported as a transmissible spongiform encephalopathy in the 1980s[1]. While it is likely the origin of CWD will never be fully elucidated, it has been postulated that CWD could have arisen as a result of sheep scrapie crossing the species barrier[2–4] to infect the mule deer or perhaps was the result of a spontaneous case that was somehow transmissible from animal to animal[5]. Because the origins of CWD are unknown and to test the assumption that all CWD originated from the initial Colorado outbreak, we sought to characterize CWD isolates from across the US to ascertain the level of strain diversity and distribution. During that study, we found some differences between states and identified isolates of interest to further investigate (Chapter 3). Continued assessment of CWD strains is necessary to better understand CWD among cervids and accurately assess threat of zoonotic transmission[6–9].

As a part of the strain typing study, two CWD-positive deer brains were submitted from Texas Parks and Wildlife for our prion strain typing analysis (Chapter 3). These two samples were initially

analyzed for inclusion in that study, but the results were so different from classical CWD isolates that they were further investigated by additional biochemical analysis, RT-QuIC and bioassay. Both isolates (TX1 and TX9) had a unique biochemical signature that suggested they might be a novel strain; however, RT-QuIC and bioassay results indicate that only TX1 had seeding potential and was infectious to transgenic mice expressing cervid PrP, but not to mice expressing HuPrP. In another twist, our negative control isolate that was inoculated in bioassay studies ended up causing RT-QuIC seeding activity and mice had PrP^{SC} in the brain that was detectable by western blot. The data presented here highlight the importance of continuing to characterize CWD strains and remaining vigilant about infectivity from isolates that present in an abnormal fashion in traditional prion assays.

Materials and Methods

Sample homogenization

Half-brain samples from two captive white-tailed deer that tested positive for chronic wasting disease (CWD) were provided frozen from Texas Parks and Wildlife. Samples were stored at -20°C until processing. Obex (TX1) or brain tissue immediately rostral to the obex (TX9) was trimmed using a disposable scalpel blade in a half of a petri dish, both were discarded after each sample. Gloves and lab bench paper were also changed between each sample. Samples were then placed in homogenizing tubes with 7-10 glass homogenizing beads and homogenized to 20% w/v in PMCA I buffer (1x PBS 150 mM NaCl, 4 mM EDTA) with cOmplete protease inhibitor (Roche). Samples were homogenized on a BeadBlaster for 2-3 rounds, with each round consisting of 3 cycles of a 30 sec pulse at 6 m/s followed by a 10 sec rest between each pulse. Samples were rested on ice for 5 min between each round. Once samples were homogenized, samples were aliquoted and stored at -20°C until further use.

PK digestion and detergent titration

For PK digestion, samples were initially diluted 1:2 in PMCA I buffer and digested in 50 µg/mL and 0.05 M EDTA for 30 min @ 37°C and 800rpm. Samples were then denatured in the presence of 3x loading buffer (200 µl 4x sample loading buffer [Invitrogen] and 80 µl of 10x sample reducing agent [Invitrogen]) for 10 min at 95°C. To optimize the digestion protocols, samples were also digested in 100 µg/mL and 0.05 M EDTA for 30 min @ 37°C and 800rpm. For detergent digestion experiments, samples were diluted 1:2 in PMCA I buffer with either sarkosyl, SDS, Triton X 100, or digitonin added so the final

concentration in the sample was equal to the concentrations noted in the results. After the detergent was added, samples were digested in 50 µg/mL PK as described above.

Western blotting

PK-digested and denatured samples were run on 12% bis-tris gels [NuPage] in 1x MOPS running buffer and transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was reduced by blocking the membranes in 5% nonfat dry milk and 1% tween-20 in 1x PBS (NFDM) for 1 hour with rocking at room temperature. Membranes were then incubated in HRP-conjugated anti-PrP monoclonal antibody Bar224 (Cayman Chemical) diluted to 1:20,000 in SuperBlock (Thermo Fischer) overnight at 4°C. Blots were washed the following day in PBST (0.2% Tween20 in 1x PBS) six times for 5 minutes each wash. Membranes were developed using enhanced chemiluminescent substrate (Millipore) for 5 minutes before imaging on ImageQuant LAS 4000 (GE). To compare between PRC1 and Bar224 immunoreactivity, blots were initially probed with HRP-conjugated PRC1 diluted 1:5000 in SuperBlock overnight and washed and imaged as above. The next day, blots were stripped in Restore Western Blot Stripping Buffer following the manufacturer's instructions (Thermo Scientific). Membrane was then blocked again in 5% NFDM for 1h at room temp and then reprobed in Bar224 1:20,000 in SuperBlock as above. Blots were washed, developed and imaged the following day as described above.

Real-Time Quaking Induced Conversion (RT-QuIC)

Because the biochemical data was so surprising, RT-QuIC analysis was performed to determine if samples had seeding activity. Samples were processed by RT-QuIC as described by Denkers *et al* (2020)[10]. Briefly, 2 µl of sample diluted to 10^{-5} in 0.1% SDS was added to 96 µl of substrate (0.10 mg/ml rPrP, 10 µM thioflavin T (ThT), 320 mM NaCl, 1mM EDTA and 1X PBS) and processed for a total of 62.5 h at 42°C on a shaking fluorimeter as described. Where noted, iron oxide bead extraction was also performed (as in Denkers *et al*., 2016) to concentrate prions before addition into RT-QuIC to detect very low levels of prions[11]. At least 8 replicates for each sample were run over two separate reaction plates. Data are shown as lag time, which represents the time (in hours) it takes the sample fluorescent signal to exceed 5x the standard deviation of the average baseline fluorescence. Some data is also shown as rate, which is 1/time to threshold.

Bioassay

To assess if TX isolates were infectious, bioassay was performed using Gt226Q mice, which express normal levels of white-tailed deer prion protein in the brain and peripherally[12]. TX1, TX9 and 18-338 brain homogenate was UV irradiated for 30 min and diluted in 1% pen/strep before inoculation. TX1, TX9 and 18-338 were used for bioassay. 18-338 was an isolate that was gifted to us from the USDA and had no detected prions by the USDA and tested negative by RT-QuIC at CSU (Table 3.8, discussed in Chapter 3). Mice were anesthetized to effect using isoflurane gas. Once mice were anesthetized, they were intracerebrally (i.c.) inoculated into the coronal suture with 30µl of 1% brain homogenate. Mice were monitored for any ill effects immediately after inoculation. Mice were monitored weekly for signs of prion disease, including tail rigidity, extensor reflex, akinesia, ataxia, tremors and hyperactivity. Once animals scored a composite score of a 9 or 10 or scored a terminal score in any category using our animal scoring system (outlined in Bender *et al.*), mice were euthanized using CO_2 inhalation[13]. Half of the brain hemisphere was frozen at -20°C and the other half was fixed in 4% paraformaldehyde for paraffin embedding and immunohistochemistry analysis. Gt226Q mice were a generous gift from Dr. Glenn Telling[12]. All animal work was performed in accordance with protocols approved by the IACUC at CSU.

For bioassay using Tg33 mice and HuPrP mice, TX1 and TX9 samples were sent to our collaborators at the NIH where Tg33, HuPrP and PrPKO mice were i.c. inoculated, monitored for disease outcome, euthanized and analyzed by RT-QuIC [14,15]. Brain homogenates from TX1-infected Tg33 mice were submitted to CSU for western blot and IHC analysis.

Immunohistochemistry

Brain hemispheres from Gt226Q mice were fixed in 4% PFA and then added to 70% ethanol before being paraffin embedded by the veterinary diagnostic lab at CSU. Then, 5 mm sections of tissue were mounted onto SuperFrost glass slides (VWR). Slides were deparaffinized and rehydrated. Then, antigen retrieval was conducted in 0.05 M sodium citrate buffer at 95°C for 20 min. Endoperoxidases were then inactivated in 0.3% H_2O_2 and avidin and biotin blocking was performed for 15 min each (Vectastain). Slides were washed in TrisA/BSA (2% BSA and 2% Triton X 100 in TBS) before blocking in 1% serum in TrisA/BSA for 1 hour at room temperature. Then, slides were incubated in 1:250 Bar224 or 1:100 GFAP overnight at 4°C. Samples were washed in TrisA/BSA 3x before blocking in 1:250 HRP-

conjugated secondary antibody for 1h at room temperature. Slides were then incubated in ABC (Vectastain) for an hour at room temperature before washing and staining with DAB (Vector Laboratories). Finally, slides were counterstained with hematoxylin and bluing reagent before being dehydrated and imaged (Olympus BX51).

Results

Texas isolates are extremely PK resistant

Samples from two male, 3.5-year-old, farmed, white-tailed deer were submitted for biochemical strain typing analysis. Both animals were 96GG and tested positive for CWD on lymph routine lymph node bioassay and were confirmed positive in obex tissue (B. Dittmar, personal communication). The remainder of the brain samples were submitted to us at CSU for strain typing analysis. TX1 brain still had a small amount of residual obex tissue that was used to make homogenates but for TX9 there was little obex so brain tissue just rostral to the obex was collected for analysis.

Because the PK-digestion protocols had worked so well for the lymph node samples that had been submitted from two previous sites that were successfully analyzed by biochemical strain typing analysis, the same PK digestion protocol was employed that utilized 50 µg/mL PK on 10% w/v sample homogenates. The experiments using 50 µg/mL PK quickly demonstrated that there was an incomplete digestion and no band shift in either of the TX samples, but there was nearly complete digestion in Tg5037 mouse normal brain homogenate (NBH, which overexpresses elk PrP ~5 fold higher than wild type brains)[16] and nearly compete digestion of PrP^{Sc} from E2, a reference strain in the lab used as a positive control (Figure 4.1 A). While there was some residual PrP^{C} signal in the NBH, this is often observed in the lab in samples after a freeze thaw (which this sample had been frozen and subsequently thawed), perhaps because of a change in the structure of the protein. We think that this structural change to an “intermediate” conformation coupled with the overexpression in this transgenic mouse line is what contributes to this incomplete digestion. Because the signal from the TX isolates could not be eliminated using 50 µg/mL PK even though the E2 samples were almost entirely digested, experiments were set up using 100 µg/mL PK (Figure 4.1 B). While there was less signal in the PK digested samples overall that when digested with 50 µg/mL PK, there was still residual signal that was PrP^{C}-like.

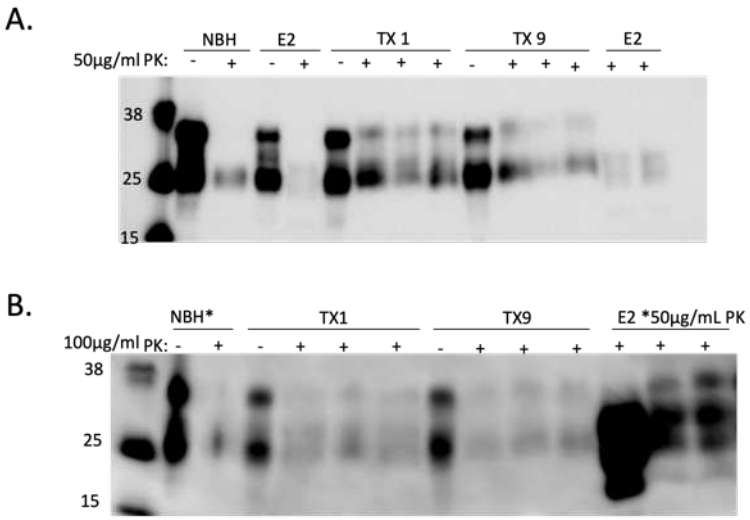

Figure 4.1. TX isolates were unable to be completely digested without the addition of a detergent. Samples were either digested in 50 µg/mL PK (A) or 100 µg/mL PK (B) in PMCA I buffer and subjected to western blotting. The asterisks in B indicate those samples were PK digested in 50 µg/mL PK. E2 = reference elk brain homogenate used in the lab, NBH = normal brain homogenate from a Tg5037 mouse.

Isolates from TX behave strangely in the presence of ionic and non-ionic detergents

To try and digest these seemingly very PK-resistant prion isolates, detergent was added to the PK digestion reaction because detergents promote the enzymatic activity of PK and are believed to break apart prion aggregates for better digestion. Sodium dodecyl sulfide (SDS), an ionic detergent, was added into the PK digestion reaction at different concentrations to determine if there would be an ideal concentration that would permit PK digestion of the TX samples. Interestingly, we found that the samples were still highly PK-resistant to SDS even after the addition of 1% SDS to the reaction mixture (Figure 4.2). This was surprising because E2 was entirely digested after the addition of 0.5% SDS to the reaction mixture, meaning that the addition of that detergent was powerful enough to digest even the PK-resistant core of the E2 prion, but not the TX isolates (Figure 4.2). This result was very surprising and suggested that the TX isolates were highly PK-resistant and not showing evidence of a band shift even after the addition of a harsh, ionic detergent.

The results from the addition of SDS indicated that SDS in the reaction would not be sufficient to digest the TX isolates, so another ionic detergent was tried. Sarkosyl was added to the PK digestion reaction in an attempt to facilitate the PK digestion of the TX isolates (Figure 4.3). As with the addition of SDS, there was incomplete digestion of the TX isolates after the addition of sarkosyl. There was a

reduction in signal of the TX isolates, but they were still present and had an electrophoretic pattern of PrP^{C}. The signal of reference isolate E2 decreased slightly in a dose-dependent manner in increasing concentrations of sarkosyl, but the signal was not eliminated as was observed with SDS (Figure 4.2)

Because the addition of two different ionic detergents did not result in a successful PK digestion that resulted in an electrophoretic band shift or elimination of the signal, a nonionic detergent, digitonin, was added to the digestion reaction as before. Once 0.5% digitonin was present in the digestion reaction, there was no detectable PrP signal in either of the TX isolates, but the E2 signal was robust and the electrophoretic pattern looked as expected for PrP^{Sc} (Figure 4.4).

To determine if this finding was consistent with another non-ionic detergent, Triton-X 100 was added to the reaction (Figure 4.5). Interestingly, the signal from the TX isolates was entirely eliminated by 0.8% Triton-X 100 in the reaction, but the signal of E2 was strong, and appeared to get stronger, in increasing concentration of Triton-X 100. By the time these experiments had begun, samples from captive white-tailed deer from deer farms across the country were provided by the USDA (Table 3.8). While E2 has been used and studied in our lab for a long time and serves as a great reference sample, the E2 isolate is from a farmed elk and not a white-tailed deer, thus the differences we were seeing may be the result of a species issue. Interestingly, with the addition of our 19-9 isolate as a control sample we saw that around 0.5% Triton-X 100 added into the digestion reaction resulted in a clean digestion of the sample where there was complete digestion of PrP^{C}, but clear PrP^{Sc} signal with an expected band shift. These results indicated that the TX isolates were not behaving as expected for a prion and, in fact, were behaving more like PrP^{C} than PrP^{Sc} in the Triton-X 100 digestion experiments.

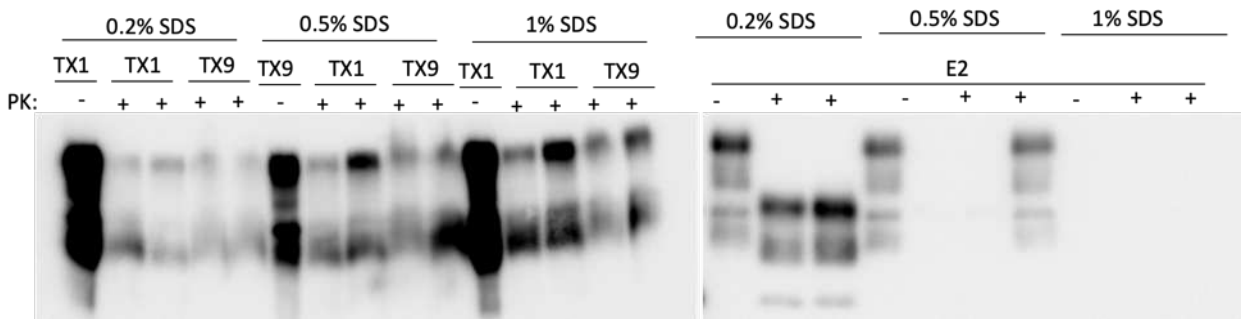

Figure 4.2. TX isolates are unable to be digested or show a stereotypical band shift in the presence of SDS. Texas isolates (A) or our reference control E2 (B) were digested in 50µg/mL PK in increasing concentrations of SDS. Even after the addition of 1% SDS there was incomplete digestion or a band shift in the TX isolates. Even the PK-resistant portions of E2 were able to be completely digested in the presence of 0.5% and 1% SDS.

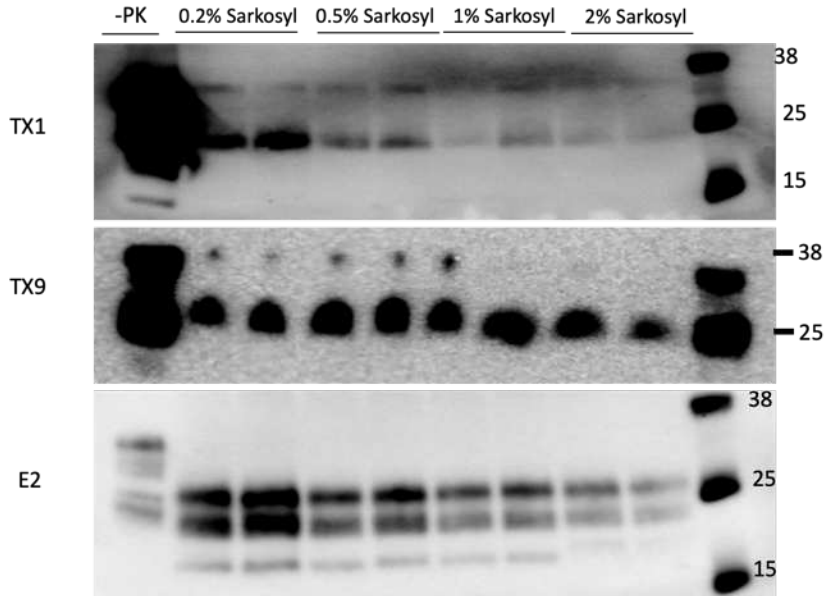

Figure 4.3. TX isolates are unable to be fully digested or show a typical in the presence of Sarkosyl. TX1, TX9 and E2 were all digested in increasing concentrations of sarkosyl.

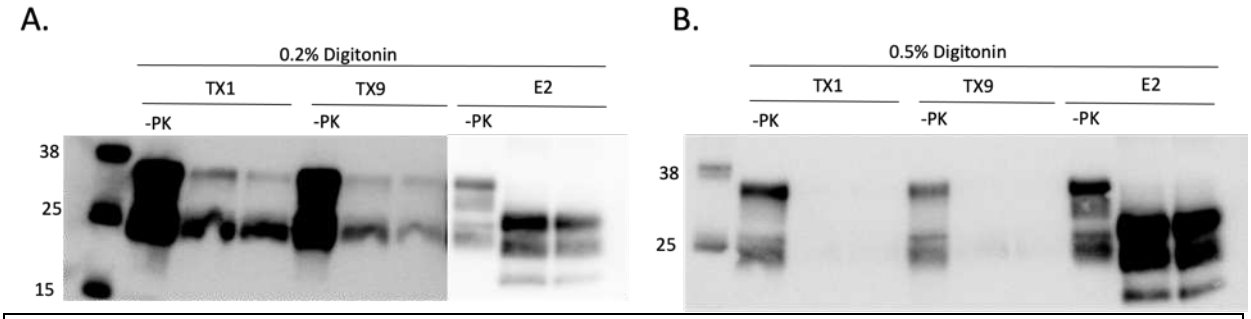

Figure 4.4. TX isolates are completely digested in the presence of 0.5% digitonin. When Digitonin was added to the sample reactions. TX samples did not show a band shift or complete digestion in the presence of 0.2% Digitonin (A). TX isolates were entirely digested in 0.5% digitonin, but E2 digested and migrated as expected (B).

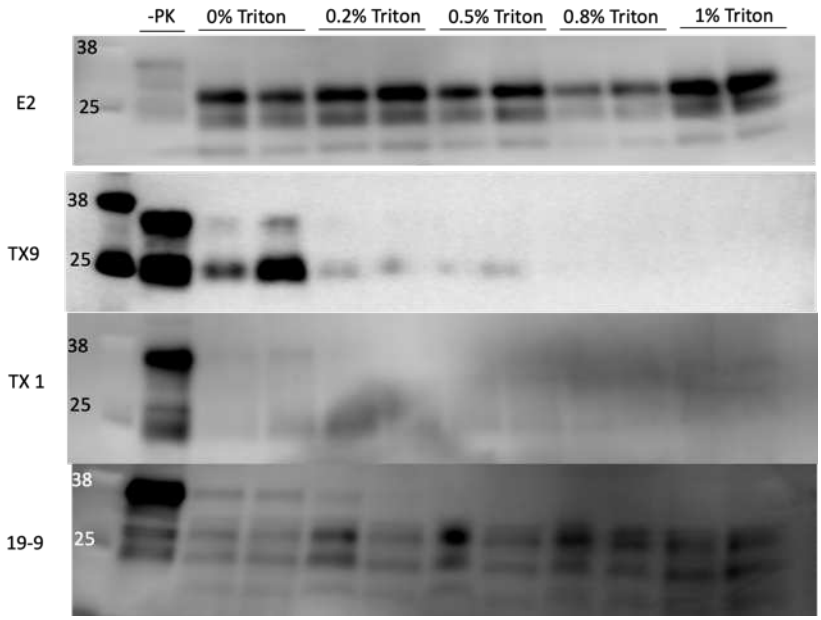

Figure 4.5. TX isolates are fully digested with no evidence of a band shift in the presence of Triton-X 100 in a dose-dependent manner. Digestion of E2 in the presence of Triton-X 100 didn't change the electrophoretic mobility. Digestion of 19-9 in increasing concentrations of Triton-X 100 improved the signal by eliminating an undigested portion of the sample by 0.5% and causing no obvious changes to the electrophoretic pattern. Both TX1 and TX9, however, had a complete loss of signal and, in TX9, this loss of signal is in a clear, dose-dependent manner.

PK-digested TX isolates are recognized by anti-PrP antibody Bar224, but not by PRC1

The interesting results from the detergent digestion experiments led us to ask if the results were the result of the antibody being used (Bar224). To ensure the results that we were seeing were not the result of antibody artifact, PRC1, another cervid-specific, anti-PrP antibody, was used[17]. For these experiments, membranes were probed with PRC1, developed and imaged. To see if the signal would be different between PRC1 and Bar224 on the same blot, the membrane was then stripped and reprobed with Bar224. Interestingly, there was no signal in the PK-digested samples when the sample was probed with PRC1[17], but there was again PK-resistant, PrP^{C}-like material in the PK-digested when the blot was stripped and probed with Bar224 (Figure 4.6). At the time of these experiments, no additional white-tailed deer brains had been procured, so a lymph node isolate from a white-tailed deer was included on the blot to make sure that any observed differences was not a white-tailed deer vs elk issue with the antibody. Both digested and undigested signal for both E2 and the lymph node isolate were detected at expected molecular weights with both antibodies, indicating the results were not due to a species difference (Figure 4.6).

Only TX1 Showed Evidence of Seeding Activity in RT-QuIC

While the TX samples were submitted as confirmed CWD positive samples, the biochemical results were surprising. To verify that the samples had prions with the ability to seed a reaction, samples were amplified using RT-QuIC. Because all of the biochemical data suggested that the results for TX1 and TX9 were the same, or at least very similar, it was surprising that RT-QuIC indicated that only TX1 had seeding activity. TX1, 19-9 and the positive control all had excellent seeding activity, but TX9 is entirely negative.

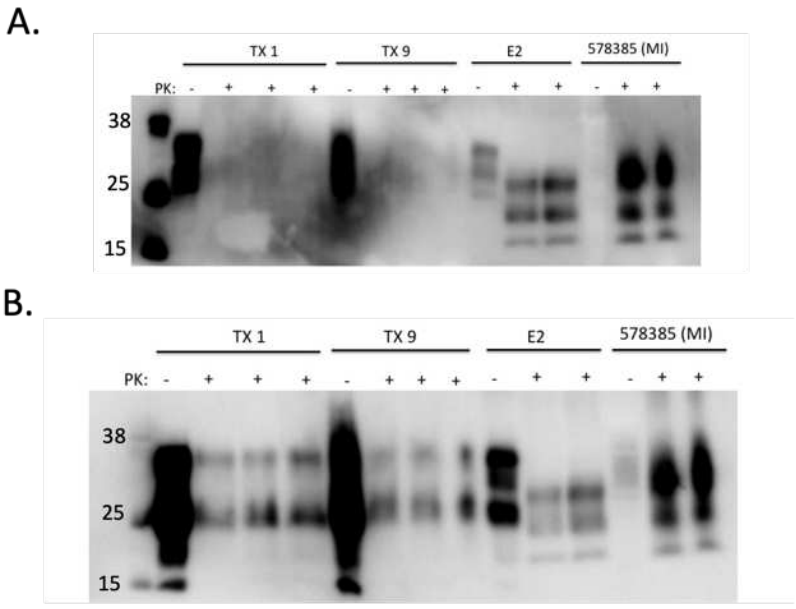

Figure 4.6. TX isolates have different reactivity when probed with anti-PrP antibodies, PRC1 or Bar224. To ensure the results we were seeing weren't an artifact of the antibody that was used, blots were probed with PRC 1 (A), the blot was stripped and then reprobed with Bar224 (B). When probed with PRC1, there is no signal in either of the TX isolates when PK digested, but there is a strong signal in the undigested samples. E2 gave strong signal in both the undigested and digested samples when probed with both PRC1 (A) and Bar224 (B). 578385 is CWD positive lymph node sample from a white-tailed deer that was included on this blot to ensure that the differences observed were not simply due to differences in between white-tailed deer and elk. There is strong signal for PK digested samples when probed with both PRC1 and Bar224.

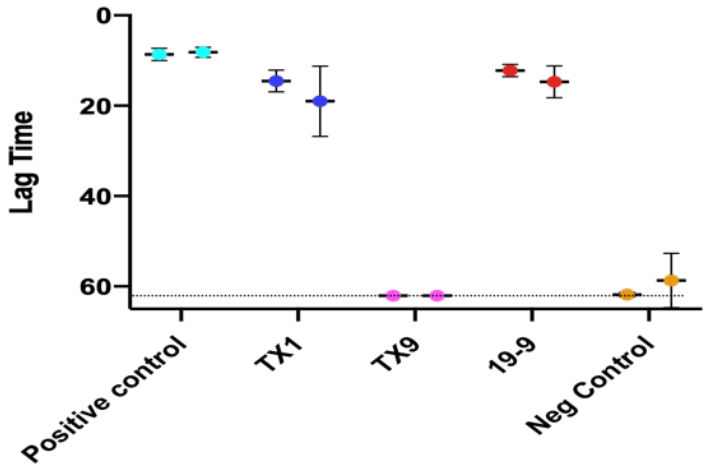

Figure 4.7. TX1, but not TX9, has seeding activity in RT-QuIC. Samples were analyzed by RT-QuIC to determine if there was any seeding ability in either of the TX isolates. TX1 showed strong ability to seed a reaction, whereas TX9 was unable to seed an RT-QuIC reaction. Results are shown in lag time, which is the time it took the samples to reach five-times the standard deviation of the background fluorescence. Dotted line shows the end of the reaction time. For each isolate, data points represent mean ± SD from two independent experiments of 4 replicates each.

Bioassay

The interesting biochemical and RT-QuIC data indicated that a bioassay into transgenic mice was the best course of action to determine if the isolates were infectious and caused a unique disease presentation (Figure 4.8, Table 4.1). As a preliminary investigation, Gt226Q mice were used. TX1, TX9 were intracerebrally (i.c.) inoculated into 5 mice/isolate and 3 mice were inoculated with negative control isolate, 18-338. Animals were monitored for signs of clinical prion disease and euthanized once they showed signs of terminal prion disease. While the bioassay in Gt226Q mice is ongoing, 3 TX1 and 2 TX9-inoculated mice were euthanized between 249-259 dpi, in accordance with our euthanasia protocols, after presenting with symptoms of terminal prion disease (Figure 4.9 A). One of the 18-338 mice was euthanized at 259 dpi, around the time the other mice were euthanized as an age-matched control (Figure 4.9 A). Upon further examination of the mice, there was some evidence of PrP^{Sc} deposition in parts of the brain and prominent astrogliosis in all mice that were inoculated with any of the isolates (Figure 4.10 I-X). The TX1 and 18-338 inoculated mice had PK-resistant PrP^{Sc} in the brain by western blot, but TX9-inoculated mice did not (Figure 4.11 A and B, Table 4.3).

In addition to the bioassay in the gene targeted mice, a mouse bioassay was also performed by our collaborators at the NIH in Rocky Mountain Labs (Figure 4.8). In this bioassay, Tg33 mice and HuPrP mice were i.c. inoculated with both TX1 and TX9[15,18]. They also ran RT-QuIC to test for seeding potential in the TX1, TX9 isolates directly, as well as on brain samples from inoculated mice (Figure 4.8). In the Tg33 bioassay, all of the Tg33 mice inoculated with TX1 developed clinical signs and died between 293-356 dpi, but none of the mice inoculated with TX9 developed clinical signs or had any evidence of prion infection by RT-QuIC (Figure 4.9 C, Table 4.2). The TX1-infected mice also all had evidence of PrP^{Sc} in the hippocampus by IHC and had evidence of astrogliosis (Figure 4.10 A-H). Mice also had PrP^{Sc} by western blot analysis (Figure 4.11 C & D). None of the HuPrP mice developed any clinical signs or evidence of prion disease/infection (Figure 4.9 B, Table 4,2).

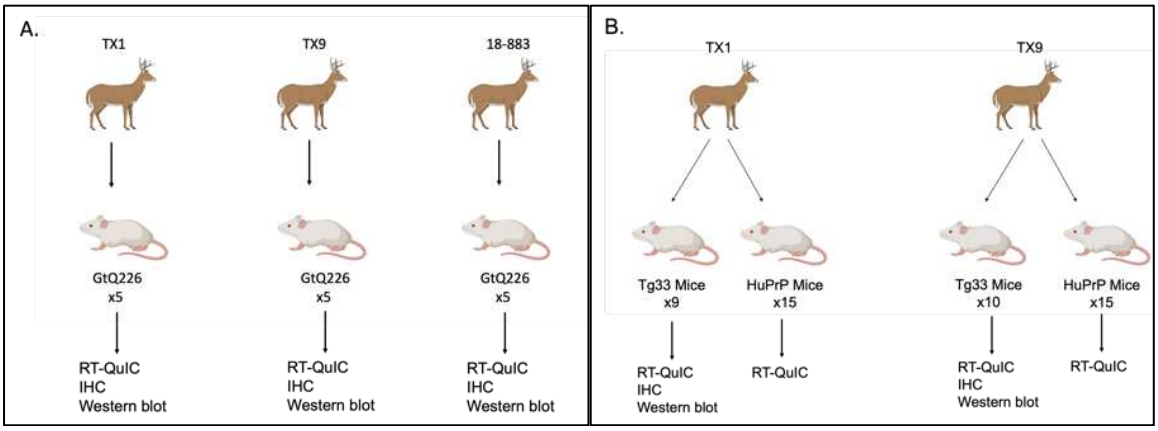

Figure 4.8. Graphical depiction of bioassay experiments on Texas isolates. Two independent bioassay experiments were set up. The first bioassay was set up as a pilot study at CSU using the new gene targeted mice (A). An additional bioassay was set up by collaborators at the NIH at Rocky Mountain Labs (B). The NIH bioassay included another transgenic mouse line that overexpressed mule deer PrP, as well as mice expressing human PrP to determine if these isolates with unique biochemical features had any zoonotic potential.

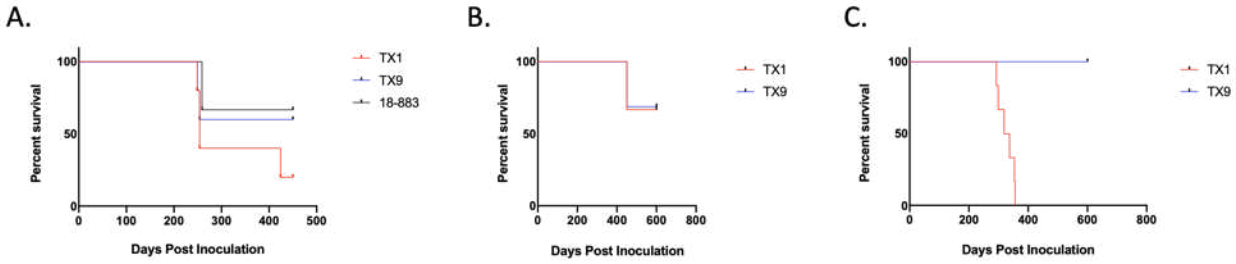

Figure 4.9. Survival curves of transgenic mice infected with TX1 or TX9. Survival curve for TX1, TX9 and 18-338 in GtQ226 mice (A), TX1 and TX9 in HuPrP mice (B), or TX1 and TX9 in Tg33 mice (C). Bioassay experiments in GtQ226 mice inoculated with 18-338 (N=2) and TX-9 (N=3) are still ongoing.

Table 4.1. Summary of Gt226Q mouse bioassay results.

	18-883	TX1	TX9
Number inoculated	3	5	5
Number euthanized	1	4	2
Clinical signs	No	Yes, 4/5	Yes, 2/5
Average DPI*	259	296±86	252±4
Spongiosis	No (1/1)	No (3/3)	No (2/2)
PrP^{Sc} positive	No (1/1)	Yes (1/3)	No (2/2)
GFAP positive	Yes (1/1)	Yes (3/3)	Yes (2/2)
RT-QuIC Positive	Yes	Yes	No

*only of mice that have shown clinical signs to date

Table 4.2. Summary Tg33 and HuPrP bioassay.

Assay	Tg33		HuPrP mice	
	TX1	TX9	TX1	TX9
RT-QuIC	9/9	0/5*	0/5*	0/5*
IHC	Positive	NA	NA	NA
Western blot	Positive	NA	NA	NA
DPI (Range)	293-356	Ongoing^	Ongoing^	Ongoing^

*Mice were euthanized at 408 dpi to survey for seeding activity
^bioassay in ongoing, but no signs of prion disease have been observed to date (>600 dpi)
NA=not available, slides were not submitted for analysis at CSU

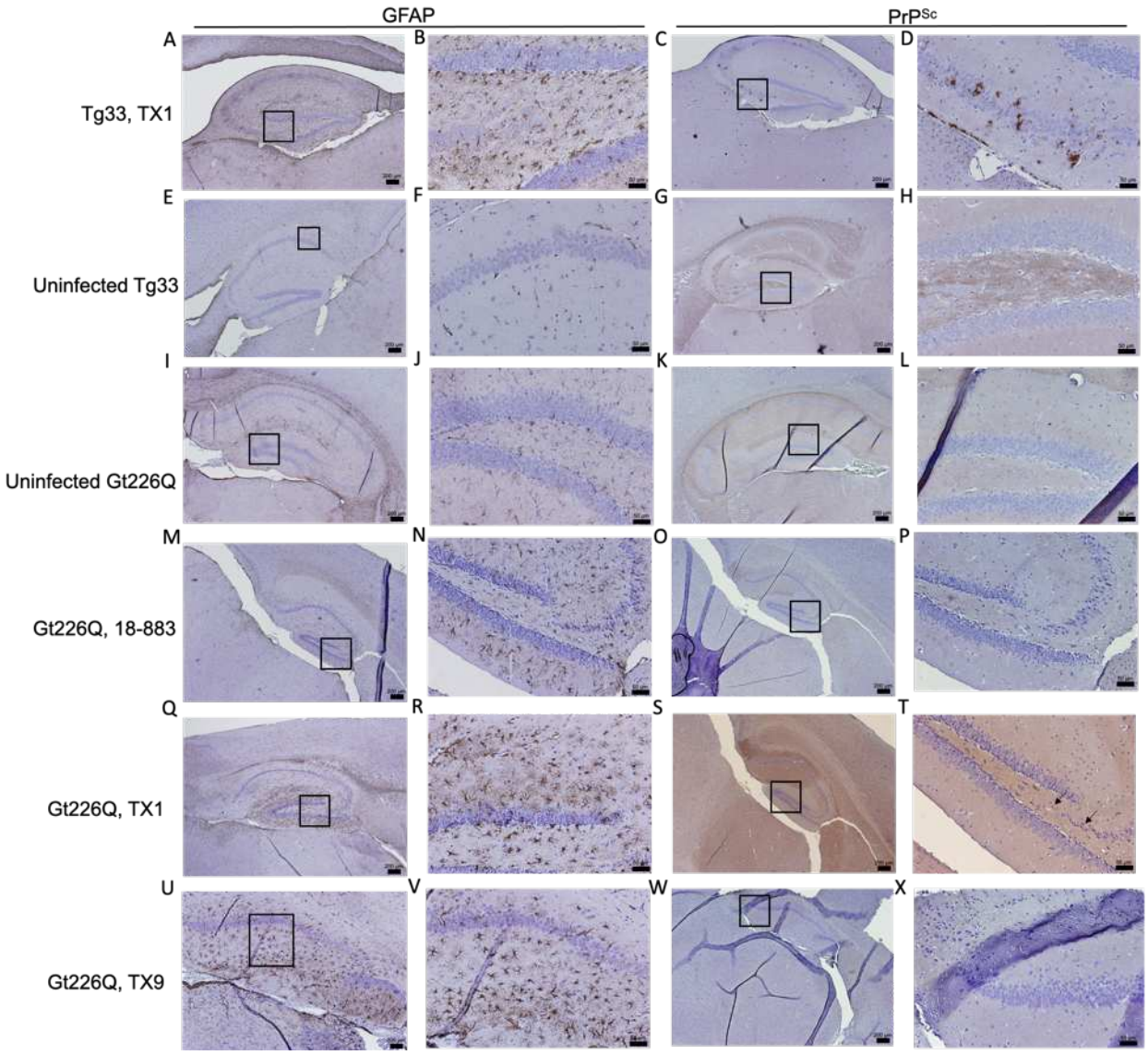

Figure 4.10. Mice inoculated with TX1, TX9 and 18-338 all have reactive astrocytes, but only Tg33 mice inoculated with TX1 have PrP^{Sc} staining in the hippocampus. Representative images for Tg33 GFAP or Bar224 in either infected (A-D) or uninfected (E-H) mice. Representative images for Gt226Q mice either uninfected (I-L), infected with 18-338 (M-P), TX1 (Q-T) or TX9 (U-X) are shown. Black boxes indicate the area that is shown at a higher magnification. Arrows are pointing to points of PrP^{Sc} accumulation when necessary, to highlight. Scale bars show 200 µm (A, C, E, G, I, K, M, O, Q, S, W), 100 µM (U) or 50 µM (B, D, F, H, J, L, N, P, R, T, V, X).

Table 4.3. Sample numbers used for running Western blot

Number	Mouse line	Inoculum
1	Gt226Q	TX9
2	Gt226Q	TX9
3	Gt226Q	TX1
4	Gt226Q	TX1
5	Gt226Q	TX1
6	Gt226Q	18-883
7	Tg33	Elk CWD
8	Tg33	TX1
9	Tg33	TX1
10	Tg33	TX1
11	Tg33	TX1
12	Tg33	WTD CWD
C1	Gt226Q	WTD CWD
C2	Gt226Q	Uninfected
C3	Tg33	Uninfected
C4	Tg33	Uninfected

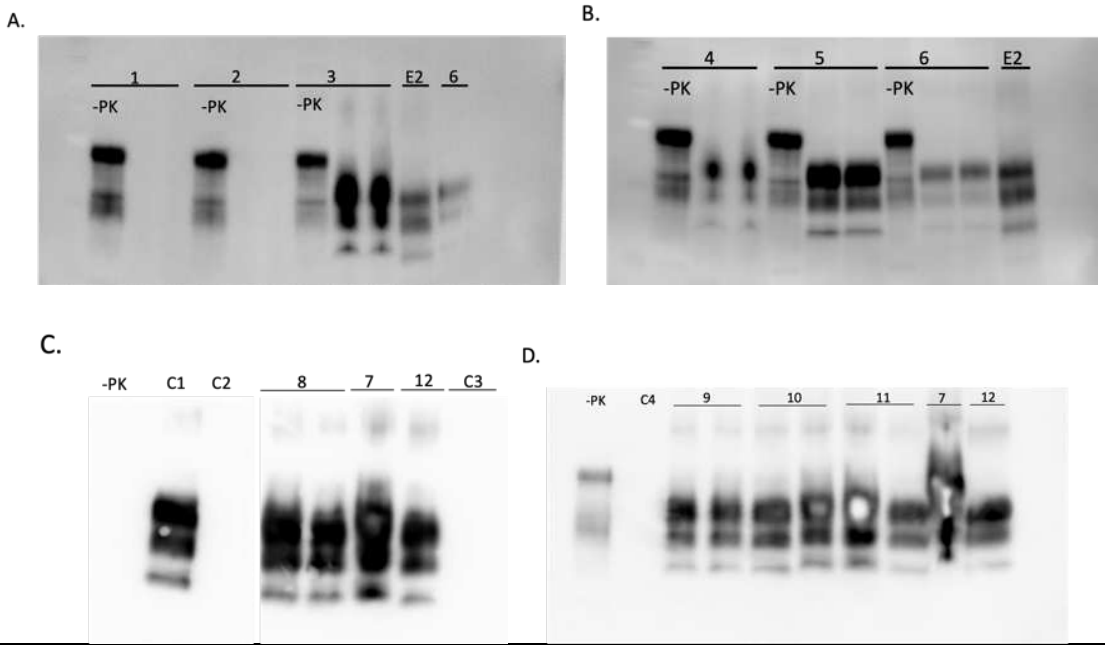

Figure 4.11. Gt226Q and Tg33 mice infected with TX1 or 18-338 had PrP^{Sc} in the brain, but mice infected with TX9 did not. Representative western blot images of animals from Gt226Q mice (A and B) or Tg33 mice (C and D). Sample identification numbers are summarized in Table 4.3.

The interesting bioassay results indicating positivity in the 18-338-inoculated Gt226Q mice and not TX9-inoculated Gt226Q mice led us to investigate the seeding potential of the inoculated mice. We hypothesized that the TX9 mice may have had prions with seeding potential that were not detectable by western blot. To assess seeding ability, isolates from TX9, TX1, 18-338 -inoculated Gt226Q mice were analyzed by RT-QuIC (Figure 4.12). In agreement with the Western blot data, only the TX1 and 18-338 -inoculated mice had seeding potential (Figure 4.12). The positivity of the 18-338 sample both by western blot and RT-QuIC begged the question if the wrong sample was used or if there was very low levels of prions in the original inoculum. To answer this question, RT-QuIC on samples either neat or after iron-oxide bead extraction. These results showed no seeding activity from the TX9-inoculated Gt226Q mice and low level, but evident seeding ability in 18-338. While there is some seeding ability in the TX9, the rate at which they come up corresponds to ~50 hours in the reaction. This late, low level signal is often not considered positive, but is somewhat suggestive that there was very low levels of seeding activity in the original TX9 inoculum (Figure 4.13).

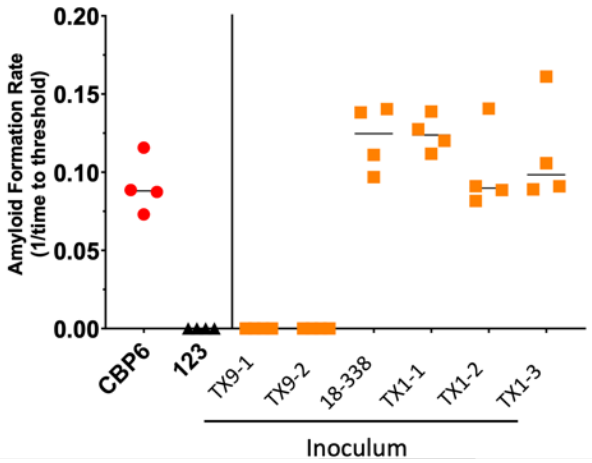

Figure 4.12. RT-QuIC seeding activity was detected in brain hemispheres from Gt226Q mice inoculated with TX1 and 18-338, but not TX9. Labels on the graph represent inoculum used to inoculate Gt226Q mice and the mouse number. CBP6 = cervid brain pool 6, a positive control, 123 = negative deer brain.

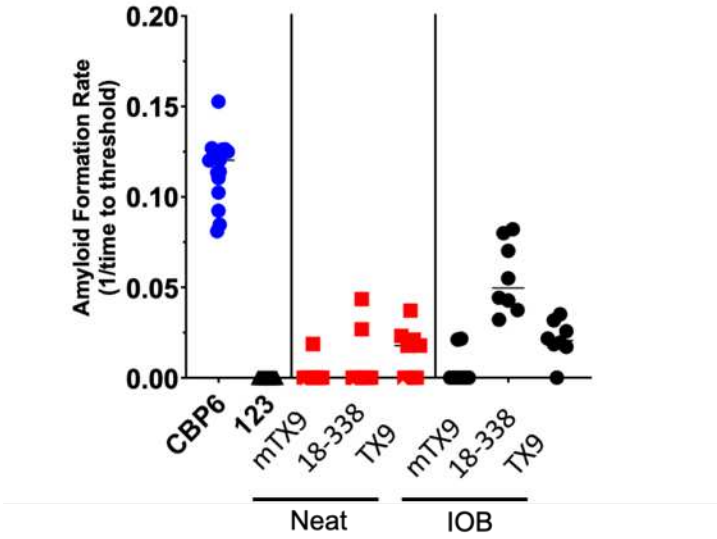

Figure 4.13. Iron oxide bead extraction revealed low levels of seeding ability in 18-338 and negligible levels in TX9, but not in brains from Gt226Q mice inoculated with TX9. To determine if the inocula had low level seeding ability that wasn't detected previously, iron oxide bead extraction was performed prior to RT-QuIC. 18-338 showed low level positivity after iron oxide bead extraction. CBP6 = cervid brain pool 6, a positive control, 123 = negative deer brain, mTX9 = brain sample from TX9-inoculated Gt226Q mouse. IOB = iron oxide bead extracted.

Discussion

Samples were submitted to us from Texas Parks and Wildlife for typical strain typing analysis as discussed in Chapter 3. The two deer submitted were both captive 3 ½ year old male white-tailed deer that were both 96GG. It was expected that the data would be generated and incorporated for our strain typing work in a week or two, but the samples behaved in novel and surprising ways that indicated there was something very interesting going on with these samples and the deer from which they were collected. This data suggested that these deer may have a novel strain of CWD.

When working with these samples, we first noticed the samples were extremely PK-resistant when digested in either 50 or 100 µg/mL PK and the electrophoretic pattern was exactly that of PrP^{C} (Figure 4.1). These findings indicated that the samples were not being adequately digested under these conditions and were much more stable than the lymph node isolates that we had been working with. This could be the result of the prions in the brain being more stable or because there is simply more PrP^{C} and PrP^{Sc} in the brains of infected animals than what our assays were optimized for. Because samples were

so PK resistant, detergents were incorporated into the digestion reaction. Surprisingly, we found that the TX isolates remained PK-resistant even in the presence of ionic detergents SDS and Sarkosyl but were entirely digested in the presence of nonionic detergents Triton-X 100 and Digitonin. These results were unexpected because ionic detergents are quite harsh and are able to disrupt all biological interactions, including protein-protein interactions. We would have expected to see either a band shift or complete digestion with these harsh detergents. Nonionic detergents, which disrupt lipid-lipid and lipid-protein interactions, were able to render the isolates so PK-sensitive that they were entirely digested in when processed with these nonionic detergents. The results we saw from E2 were more of what we expected to see when adding these two different detergent classes to the reaction, with SDS being able to entirely eliminate the signal. 19-9 also behaved more in line with what is expected of a traditional prion disease, where the addition of some detergent enabled for a complete digestion of PrP^C and a cleaner signal. These data suggested that there was something structurally or conformationally unique about these prion isolates that were influencing protein behavior with different detergent classes. Perhaps there was a lipid-protein interaction that needed to be disrupted with the nonionic detergents for the isolates to be digested adequately.

To ensure that the surprising results from the PK-digestion and the detergent digestion experiments weren't an artifact from the antibody, another anti-PrP antibody (PRC1) was used. PRC1 is a cervid PrP-specific antibody that an epitope in the charged cluster of the prion protein[17] which is N-terminal to the alpha-1 globular domain recognized by Bar224. Surprisingly, there was no signal in the samples when probed with PRC1, but there was again PrP^C-like signal when stripped and probed with Bar224. It is surprising that only the PK-digested TX isolates are not detectable by PRC1, but the samples without PK were detected. Because the results of the reprobing with Bar224 indicate that the TX signal is still the same molecular weight as PrP^C, it is unlikely that the epitope was digested by PK and that is why it was not detected by PRC1. Western blots are run on denatured samples, so it is hard to imagine that a structural difference would be observed in this assay, but prions are thought to refold into a PrP^C-like conformation after denaturation once on the membrane[17,19]. Perhaps when these isolates are refolding on the membrane, the protein is folding into a conformation that hides the epitope of PRC1. This finding must be contingent upon PK because the undigested sample is clearly detected in the samples

without PK. If true, this would indicate that the prion in these isolates exists in a structure where the typically PK-sensitive N-terminal portion of the protein is protected from PK and the prion remains the same molecular weight as PrP^C, complicating efforts to discriminate between them. In this case, perhaps in the samples digested with PK, the "true" PrP^C is still digested, but the PrP^C-like prions that hide the PRC1 epitope remain and are subsequently detected by Bar224. Additional epitope-mapping experiments utilizing additional PrP antibodies would be helpful to get additional conformational information on these isolates.

Because all the biochemical data were so unique, RT-QuIC was employed to ensure that we weren't simply seeing incomplete digestion of PrP^C and we were dealing with was a prion that at least had seeding potential[10]. We found that only TX1 had seeding potential, which was surprising because so much of the biochemical data indicated that the data between TX1 and TX9 was nearly identical. Later investigation of the TX9 isolate using iron-oxide bead extraction of the sample also indicated that there was no seeding activity of the sample in RT-QuIC (Figure 4.13)[11]. These data suggest that perhaps the unique biochemical findings are simply due to PrP^C from these animals or perhaps that they both did have CWD, but only one of the animals had prions that had seeding activity under the conditions utilized in these experiments.

Because RT-QuIC looks at the seeding ability of a prion and seeding activity doesn't necessarily indicate infectivity, bioassay was also employed to characterize the prions from the TX isolates using traditional methods. Isolates were inoculated into mice expressing cervid PrP to assess the isolates in cervidized mice, as well as mice expressing the human prion protein to determine if these prions with bizarre biochemical characteristics had any zoonotic potential. While some of the studies are ongoing, it appears that the TX1 isolate is infectious and causes a typical CWD infection in Tg33 mice. These mice had traditional CWD plaques in the brain, spongiosis and astrogliosis (Figure 4.10) as well as PrP^{Sc} detectable in the brain by western blot (Figure 4.11). Animals also all succumbed to prion infection in a normal timeframe for Tg33 mice. TX9 inoculated Tg33 mice, however, showed no evidence of prion disease by RT-QuIC from mice sacrificed as a check at 408 dpi and none of the mice presented with clinical prion disease at the time of this writing (data performed by collaborators and not shown here). The results from this bioassay support the results from RT-QuIC that only TX1 is a bona fide prion as currently

defined. Fortunately, all of the HuPrP mice have no evidence of prion disease and are RT-QuIC negative, indicating that this prion isolate does not have any increased zoonotic potential.

While the Gt226Q bioassay is still ongoing, the data obtained from that study have been less straightforward than the Tg33 bioassay. Infected mice presented with clinical signs of prion disease around 250 dpi and were euthanized in accordance with our disease scoring and IACUC protocols (Table 4.1). Upon IHC examination, it was found that all the mice had some astrogliosis, but none of the mice had evidence of spongiosis and only one of the TX1-inoculated mice had evidence of PrP^{Sc} deposition, but the plaques are very small and suggest that the mouse was very early in its disease course (Figure 4.10). All of the TX1 mice had RT-QuIC seeding activity (Figure 4.12). At the time of this writing, a cage mate of the TX1 mice that were euthanized at 250 dpi is showing evidence of clinical prion disease at 430 dpi, close to what was published for this mouse strain[12]. This mouse showed no signs of disease when the others were euthanized. None of the TX9-inoculated mice had any PrP^{Sc} in the brain by Western blot or IHC and the samples were unable to seed an RT-QuIC reaction (Figure 4.12). There was no evidence of spongiosis, but both euthanized mice show signs of neuroinflammation when assessing reactive astrocytes (Figure 4.10).

While all of our evidence suggests that TX9 is not infectious, it doesn't necessarily mean that the isolate that was given to us was not a true infection. Diagnostic labs require the obex for diagnosis of CWD, it is possible that the prions were a low titer in the obex to begin with and the remaining parts of the brain, which were used in our assays, simply don't have a high enough titer to be detected in RT-QuIC or establish an infection in transgenic mice. It is still somewhat surprising that the mice that were euthanized had evidence of reactive astrocytes (Figure 4.10). It's possible that putting a homogenate from a different animal (deer into mouse) caused some inflammation. While this would be expected in the time shortly following infection, it is not likely that is what we are seeing because the animals had been inoculated ~250 days prior to euthanasia, but the possibility cannot be excluded that the inflammatory response is simply from the inoculum. It is also possible that there is another pathogen or toxin present in the deer brain homogenate that is causing neuroinflammation in our i.c. inoculated mice. Examination of the TX9-infected Tg33 mice will be helpful to determine if the neuroinflammation is an artifactual result from this mouse model or consistent across mouse strains. This will be completed once slides are received from

our collaborators. Alternatively, it is possible that both the TX1 and TX9 animals did have prion disease but contracted the disease differently. Maybe the TX9 animal is an example of non-adaptive prion amplification that isn't infectious upon serial passage or detectable in RT-QuIC when a hamster prion protein substrate is used, but would be transmissible back to the original species. More information would be required from our TX collaborators to determine if this is a viable hypothesis.

One of the most surprising findings from this study is the Gt226Q mouse inoculated with 18-338 that was euthanized to be an age-matched animal and was not presenting with any signs of terminal prion disease has data to suggest it was positive for PrP^{Sc}. This isolate was chosen as a negative control to inoculate into the Gt226Q mice because it had no detectable prions in either the obex or the lymph node (according to diagnostic results shared with us), was negative by western blot analysis and negative by RT-QuIC (Figure 3.20, Table 3.8). Surprisingly, the animal that was euthanized had astrogliosis, was positive for PrP^{Sc} by western blot and was able to seed an RT-QuIC reaction at a rate similar to the TX1-inoculated mice (Figure 4.12). After these results, the 18-338 inoculum was further analyzed for evidence of seeding activity in RT-QuIC. Indeed, after iron-oxide bead extraction there was seeding ability in the sample, albeit at a low level (Figure 4.13)[10,11]. Because CWD-positive deer show positivity in the lymph node samples before the brain, it is surprising that this animal was found negative by lymph node and obex analysis but was positive in more rostral sections of the brain, even if only at a low level[20–22]. We would have expected this animal to have a positive result in the lymph node if we were able to detect PrP^{Sc} in the brain. Perhaps this is an infectious isolate of CWD that is PK and formic acid resistant (formic acid is used to distinguish between PrP^{C} and PrP^{Sc} in IHC) so was unable to be detected by conventional methods prior to enrichment before RT-QuIC or bioassay. There is no genotype or genetic information for this animal, but it would be interesting to know if there was anything unique about the genetic makeup of this animal. The other two mice that were inoculated with 18-338 are still alive and not showing signs of prion disease to date. Analysis of these additional mice will be informative about the infectious nature of this isolate.

Taken together, the data presented here indicate that TX1 is a prion isolate with a unique biochemical signature that, once passaged into transgenic mouse models, behaves like a typical CWD isolate. Perhaps the reason the biochemical results were so surprising is because the titer in the sample

was extremely low and we were simply optimizing the best detergent to digest white-tailed deer PrP^C in our hands. It is also possible that this prion strain was unique, biochemically, in the white-tailed deer and only upon passage into mice began to behave as would be expected in the prion field. It is unclear how often this occurs because so few studies characterize the inoculum before passage into a mouse model. PK-sensitive, soluble oligomers have been shown to be more infectious than PK-resistant plaques and perhaps what we saw is the result of an animal that is early in its disease course that made it difficult to detect prions using our traditional methods[23,24]. TX9 presented similar to TX1 in biochemical assays, indicating that the prions in TX1 were more similar to PrP^C than PrP^{Sc}, perhaps because the animals were early in their disease course. While the biochemical data is quite confusing when assessing TX1 and TX9, it highlights that samples can be infectious even at a low titer or before the development of PK-resistant material biochemically present in a way similar to PrP^C. 18-338 is also an interesting finding because this sample was expected to be entirely negative. The biggest surprise there is that this animal was negative in the lymph node but was infectious in rostral brain sections. This may represent a new prion strain that is less lymphotropic than expected for CWD. If true, this would make antemortem detection of CWD even more challenging. This research highlights the importance of further assessing samples that appear to be prion negative for the presence of infectious material biochemically.

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Chapter 5:

Rice plants are able to uptake infectious prions and implicate a role for short-term, plant vectored chronic wasting disease transmission

Summary

Chronic wasting disease is a devastating transmissible spongiform encephalopathy affecting free-ranging and captive cervids worldwide. One unique characteristic of CWD is how highly transmissible it is between animals, both with direct contact and through contact with contaminated environment. To better control CWD, it is imperative that all aspects of CWD transmission be understood to better predict and understand transmission dynamics. Here, we examined the ability of rice plants to uptake infectious prions from spiked water samples. The data presented here indicate that rice plants are able to uptake prions in our system by 2 and 24 h post exposure (hpe), but there are no infectious prions detected above the 99% confidence interval at 72 hpe. These results indicate that plants are able to uptake infectious prions shortly after exposure. This data contributes to a growing body of work implicating plants as a vector potentially playing a role in CWD transmission.

Introduction

Chronic wasting disease (CWD) is an invariably fatal prion disease affecting white-tailed deer, mule deer, elk, moose, red deer and reindeer[1–9]. Experimental infections have also shown sika deer to be susceptible to prion disease, but they have not been documented cases in free-ranging animals to date[10,11]. CWD was first noted in captive mule deer from Colorado in the late 1960's[12]. Since that time, the disease has spread worldwide and has been documented across North America, South Korea[13] and, most recently, Scandinavia[2,6]. CWD is highly transmissible and once CWD has been found in a population, it becomes excruciatingly difficult to control. Horizontal transmission of prion disease is the most important mechanism for CWD transmission[14], but vertical transmission has been documented and may play a contributing role to disease transmission[15–17]. Direct contact between individuals plays an important role in transmission of CWD and prions have been found in urine, feces, blood, nasal secretions, saliva and antler velvet, providing many opportunities for prions to pass from one individual to

another[18–23]. Infected animals have been shown to shed infectious prions before they start showing clinical signs, indicating that a seemingly normal deer may be passing prions to naïve animals[24].

While horizontal transmission is a critical component of CWD transmission, mathematical models have indicated that an environmental reservoir and environmental transmission is also a critical component to CWD transmission[25]. In fact, prions are extraordinarily stable in the environment and exposure to environmental fomites or living in contaminated pens is sufficient for CWD transmission[24,26,27]. Another animal prion disease, scrapie, has been shown to remain infectious in the environment for up to 16 years after the removal of infected animals[28]. The longevity of prions in the environment is one of the greatest barriers to control of CWD once it has been introduced into a population. This longevity has led Norway to try and control CWD in its reindeer population through dramatic measures, including culling entire herds and isolating the contaminated habitat for 5 years[6].

Additional work on environmental prions have indicated that soil, specifically montmorillonite clay, binds to prions extremely tightly and these soil-bound prions are actually more bioavailable that unbound prions[29]. Water collected from CWD-endemic areas has also shown positivity and could be another avenue for environmental transmission[30]. Soil-bound prions are a relevant possibility of transmission to deer because animals will consume small amounts of soil inadvertently and intentionally as a source of minerals[31]. As animals die in nature, decaying carcasses are also able to deposit prions into the environment and predators and scavengers have been shown to pass infectious prions in their digestive system across the environment[32,33]. While other animals have been shown to spread infectivity across the landscape, activities and behaviors that bring deer in close proximity, including salt licks, deer baiting and overwintering locations, are known to facilitate CWD transmission, likely through close contact between the cervids and concentrating infectious prions in the environment for animals to be easily exposed[14,34].

In addition to an environmental component of CWD transmission, there is also the possibility of vectors playing a role in CWD transmission[31]. Plants are a critical component of cervid diets and could play a role in transmission of CWD[31]. Previous papers have looked at the ability of plants as a vector for CWD transmission. Rasmusen *et al.* found that plant roots were able to bind to recombinant PrP^C or proteinase K digested prions, but not bona fide prions. They also investigated the ability of plants to uptake prions into aerial structures but were unable to detect anything using their methods that did not

include any amplification assays[35]. Pritzkow *et al.* also examined the ability of plants to bind and uptake 263K prions and found that grass plants were able to bind prions to their external surface and remain infectious upon hamster bioassay, as well as uptake prions into aerial structures using protein misfolding cyclic amplification. Pritzkow *et al*. added critical knowledge to the field as to whether plants were even able to uptake infectious prions; however, they used an extremely large volume of a high percentage brain homogenate when exposing plants to prions that is likely not biologically relevant. They also used 263K, a scrapie prion that has been adapted to hamsters, for their plant uptake experiments and not CWD prions[36].

The goal of this work was to expand upon what has been known in the field about the interaction of plants with prions and to continue to elucidate the role of plants in CWD transmission. For these experiments, we investigated the ability of another type of plant (rice plants) to uptake prions in the absence of soil with an intact root system. Plants were also exposed to fewer prions than in previous studies (1%). Furthermore, this work is the first to look at experimental plant exposure to CWD prions. This data will be critical in our understanding of the role of plants in transmission of CWD and will inform models to help control the spread of chronic wasting disease.

Materials and Methods

Plant handling and exposures

Rice plants (*Oryza sativa* cultivar *kitaake*) were grown for 45 days by the Leach lab at Colorado State University. It was anticipated that plants would be grown hydroponically but were received having been grown in soil. Because prions have been shown to bind so tightly to prions, attempts were made to remove the soil from the roots. Plants were carefully removed from the soil and every attempt was made to not damage the root system. Plant roots were then gently rinsed in DI water to remove any additional unbound soil. Then, plants were grown in water only (plant #1), food dye (plant #2), water and food dye (plant #3), or water and food dye and 1% E2 brain homogenate (plants #4-6). E2 brain homogenate was briefly sonicated before adding to the water sample to break up any large plaques that could prevent the uptake of sample. Every caution was taken to prevent contaminating the external surface of the plant with prion material. Plants were grown for either 2, 24 or 72 h before they were removed from the water they

were grown in. Water that the plants were grown in was frozen at -80°C for future verification that prion-negative samples were indeed negative and spiked samples were positive for prions. For all plants, care was taken to change out all equipment, gloves and lab bench paper between each plant. Disposable scalpel blades were used for all plants and changed between each plant section and between each plant. After plants were sectioned, they were placed in plastic bags (Ziploc) and stored at -20°C. Later, plants were removed from the freezer, placed into Bioreba bags, weighed, and then homogenized by hand to a 10% w/v homogenate in 1x PBS. Samples were aliquoted and stored at -20°C for the short term and then transferred to -80°C for long-term storage.

PK digestion

Proteinase K (PK, Roche) was diluted in 1x PBS to either 500 µg/mL (for PMCA'd material) or 100 µg/mL (non-PMCA'd material) and 2 µl of PK was aliquoted into Eppendorf tubes. Then, 18 µl of the appropriate sample was added for a final concentration of PK in the sample equal to 50 or 10 µg/mL. Samples were incubated at 37°C and 800 rpm for 30 min on a shaking heat block. 3x loading buffer (200 µl 4x sample loading buffer [Invitrogen] and 80 µl of 10x sample reducing agent [Invitrogen]) for 10 min at 95°C. Samples were then either saved at -20°C or immediately run by western blot.

Western Blotting

Samples were run on 12% bis-tris gels (NuPage) in 1x MOPS running buffer and transferred to polyvinylidene difluoride membranes. Non-specific binding was reduced by blocking the membranes in 5% nonfat dry milk and 1% tween-20 in 1x PBS (NFDM) for 1 hour with rocking at room temperature. Membranes were then incubated in HRP-conjugated anti-PrP monoclonal antibody Bar224 (Cayman Chemical) diluted to 1:20,000 in SuperBlock (Thermo Fischer) overnight at 4°C. Blots were washed the following day in PBST (0.2% Tween20 in 1x PBS) six times for 10 minutes each wash. Membranes were developed using enhanced chemiluminescent substrate (Millipore) for 5 minutes before imaging on ImageQuant LAS 4000 (GE).

NBH Preparation

To prepare normal brain homogenate (NBH) for use in PMCA, Tg5037 mice, that overexpress elk PrP, were genotyped to ensure they were expressing elk PrP. Only mice that were positive for the elk PrP gene were included in the NBH prep.

Mice were euthanized with 1% CO2 until there were no breaths taken for 10 s and there was no response to foot and tail pinching. Once mice were clearly deceased, their fur was sprayed with 70% ethanol and the skin above the peritoneal and plural cavities was cut away. Then the sternum was snipped off with scissors and the rib cage was reflected. The left ventricle of the then identified and a butterfly needle was used to enter the left ventricle. Once the needle was in place, scissors were used to cut the right atrium. Once the right atrium was snipped, mice were perfused with 30mL perfusion buffer (5mM EDTA in 1x PBS). Once the perfusion was complete, the mouse was turned to a prone position, the skin around the head of the mouse was reflected and the head was separated from the body. Then, a rostral cut was made through the skull (beginning in the foreman magnum) down to the nose, the brain was removed, divided sagittally, placed into homogenizing tubes and kept on ice. Once weights were taken, PMCA I buffer (1x PBS, 4mM EDTA, 150mM NaCl) and cOmplete protease inhibitor tablets (Roche; 1 tablet for 5 mL of PMCA-I buffer) was added to brain sections to make a 20% w/v homogenate. Then, 4-6 glass beads were added to each tube and homogenized for one 30sec pulse. Samples were then pooled and diluted to a 10% w/v solution in PMCA II buffer (2% Triton X in PMCA I buffer). The final concentration of protease inhibitors in the final 10% NBH is 1x and the final concentration of Triton X is 1%. NBH was then aliquoted and kept at -80°C until use.

Protein misfolding cyclic amplification (PMCA)

Samples that were below the limit of detection by straight Western blot were amplified using PMCA. Samples were diluted by adding 25 µl of sample to 25 µl of Tg5037 NBH in PMCA tubes. Each sample was run in triplicate. Samples were then placed into the sonicator (Q700, Qsonica) with 300 mL of DI water such that the bottom of the tubes were a ~5 cm above the bottom of the horn. Samples were sonicated for 40 s pulses every 30 min at 40% power for 24 h (=1 round). After each round of PMCA, 25 µl of the previous round of PMCA-amplified material was diluted into a fresh 25 µl of Tg5037 NBH and sonicated for another round. E2 was run as a positive control and prepared fresh for each round to ensure the sonication and amplification was successful. NBH samples were serially run through PMCA to control for amplification and spontaneous conversion of PrP^{C} to a misfolded PrP^{Sc}. A total of 6 rounds were performed. Samples were either PK digested immediately or stored at -20°C until future use.

For PMCA using 1536 NBH (gifted from Dr. Glenn Telling's lab), 5 µl of prion seed (diluted in PMCA conversion buffer [PMCA I buffer with 1% Triton X 100]) was added to 45 µl of NBH and sonicated at 70% power every 20 s for 48 h. Only 1 round was performed.

Data analysis

Western blot data were analyzed and each sample replicate was called as either positive or negative. To semi-quantify the samples, relative PMCA units (rpu) were calculated as in Pulford *et al.* (2012)[22]. Briefly, raw scores were calculated depending on the round at which the sample first came up positive. Raw score = $(n + 1) - r_+$, where n is the total number of PMCA rounds and r_+ is the first positive round. The raw score was then normalized by dividing the raw score by the highest possible score for that sample and multiplying by 100 (Figure 5.4). Normalized scores for all three replicates were averaged for each sample, giving each sample a relative PMCA unit and standard error of the mean. Data were graphed in GraphPad Prism (Version 8.30). Confidence interval calculations and relative PMCA units were calculated in MS Excel. Confidence intervals were determined by taking the average rpu of the NBH samples plus four times the standard error of the mean.

Results

Experimental overview and verification plants were exposed to prions as expected

The experimental setup is shown in Figure 5.1. Before any analysis was conducted on plant tissue, the water that the plants were grown in were tested by western blot to ensure there was prion positivity only in the samples that were spiked with E2. Water samples from plants 1-6 for all three timepoints were tested by western blot for positivity. Across all three time points, positivity was only seen in the samples that were spiked with E2 (Figure 5.2). Importantly, E2 was spiked into the samples at a final concentration of 1% and, when PK digested in 10 µg/mL PK, run by western blot and compared to 0.1% and 0.01% E2, the signal appears lower in the plant fluid samples.

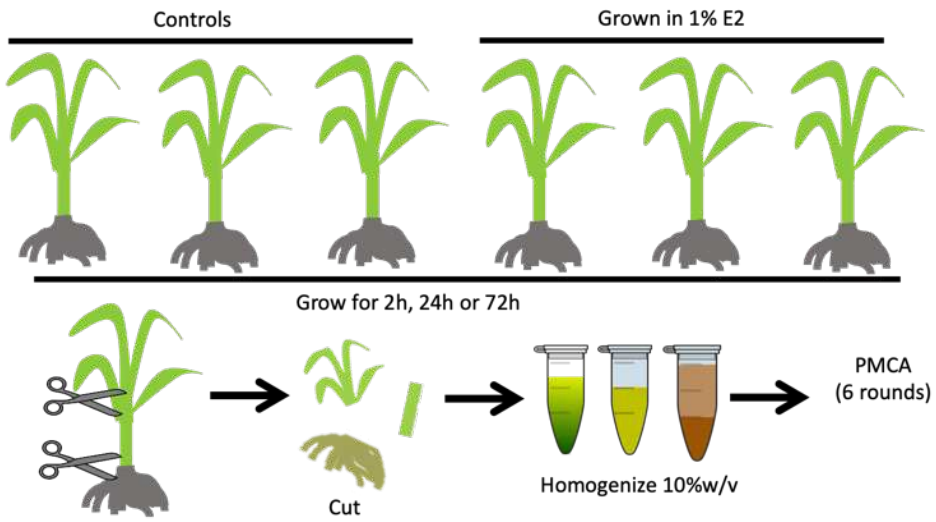

Figure 5.1. Experimental setup of plant exposure experiments. Graphical overview showing the experimental design. Cuts were made first between the roots and the stem and then between the stem and the leaves. Disposable scalpel blades were changed between each Plants were cut at the beginning of the first node and sample above that point was considered leaf material.

Prion positivity in plant samples

After verifying that plants were exposed/not exposed as expected, samples were tested before PMCA to determine if there were any detectable prions before amplification. There was no detectable PrP^{SC} in any of the samples before PMCA (Figure 5.3). After determining there were no detectable prions in the samples before PMCA, samples were subjected to a total of 6 rounds of PMCA as described in the Methods section. Samples were tested by Western blot at rounds 3 and 6 to assess any potential positivity. If any positive samples were found, previous rounds were removed from the freezer, PK digested and run by western blot to find the round at which the sample first came up positive to incorporate into the relative PMCA unit calculations (Figure 5.4). Sample PMCA blots and final results for rpu calculations are shown in Figure 5.5.

Interpretation of PMCA

While PMCA was reliable and interpretable for the duration of this study, efforts to repeat/expand on these findings were difficult because of the technical difficulties with PMCA (Figure 5.6). As shown in Figure 5.6, E2 and NBH signals became indistinguishable, making data interpretation and analysis impossible. Over 30 troubleshooting experiments were set up to address the issue to no avail (Table A.1). Because samples were below the limit of detection before PMCA and PMCA was no longer an option for

amplifying material, experiments were unable to be continued. Years later, PMCA was once again successful with 1536 NBH (1536 mice over express white-tailed deer PrP) and a white-tailed deer prion seed (Figure 5.6).

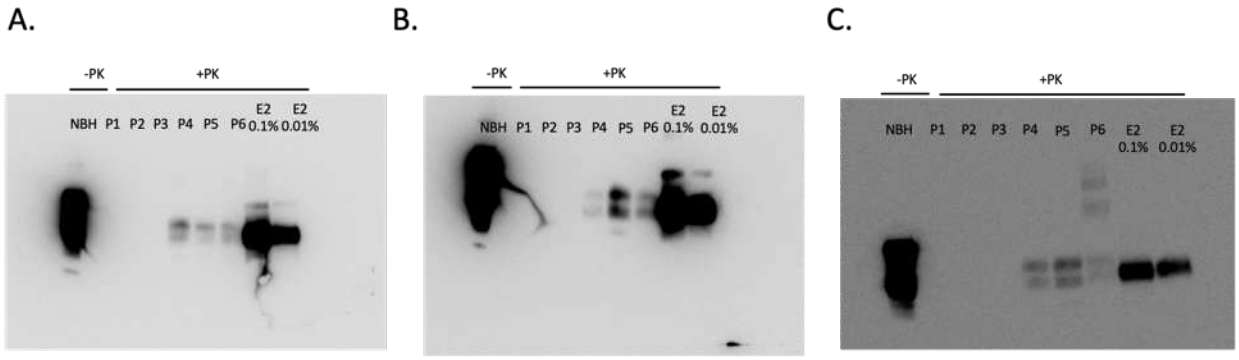

Figure 5.2. Detectable CWD prions are found in the prion-spiked water, but not in water used for control plants. Plants that were exposed for 2 h (A), 24 h (B), or 72 h (C) had test fluid examined by western blot to ensure there were detectable prions in the exposed plant samples. E2 was run 10- and 100-fold less than the amount of E2 that was used to expose the plants. Plant water and E2 was PK digested in 10 µg/mL PK and NBH was not digested with PK to show the electrophoretic migration pattern of PrP^{C}.

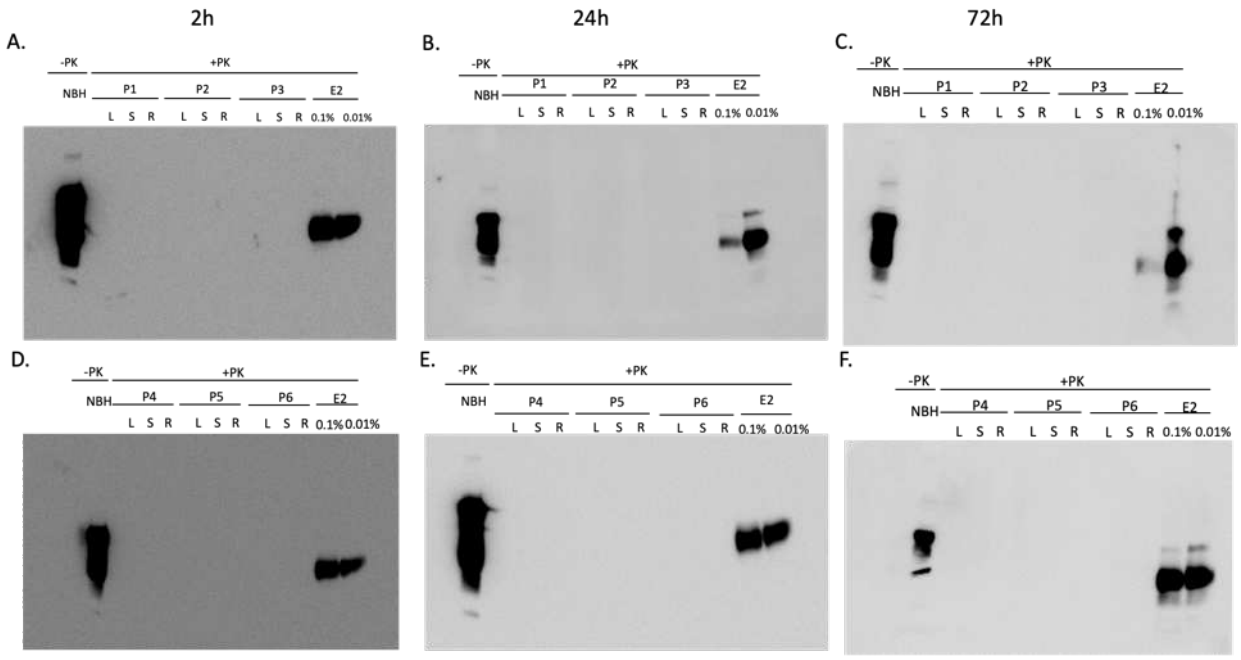

Figure 5.3. No PrP^{SC} signal is detectable in any plant tissue before PMCA. Samples were PK digested in 10 µg/mL PK except one NBH sample, which was run to demonstrate the electrophoretic pattern of PrP^{C}. No PK-resistant material was detectable by western blot in any plant tissues at 2 h (A, D), 24 h (B, E) or 72 h (C, F). The letter P followed by a number refers to the plant number. L=leaf, s=stem, r=root. E2 was run as a prion migration control.

PMCA scoring

1st (+) round	raw score	[1]normalized score
A	6	100
B	5	83
C	4	67
D	3	50
E	2	33
F	1	17
none	0	0

[1]normalized score = raw score/total possible x 100

Figure 5.4. Semi-quantitative PMCA scoring system used to calculate relative PMCA units (rpu). This table indicates how a later round of PMCA (round F, for example) being the first round to have prion positivity results in a lower normalized score. Figure modified from Pulford *et al.* (2012).

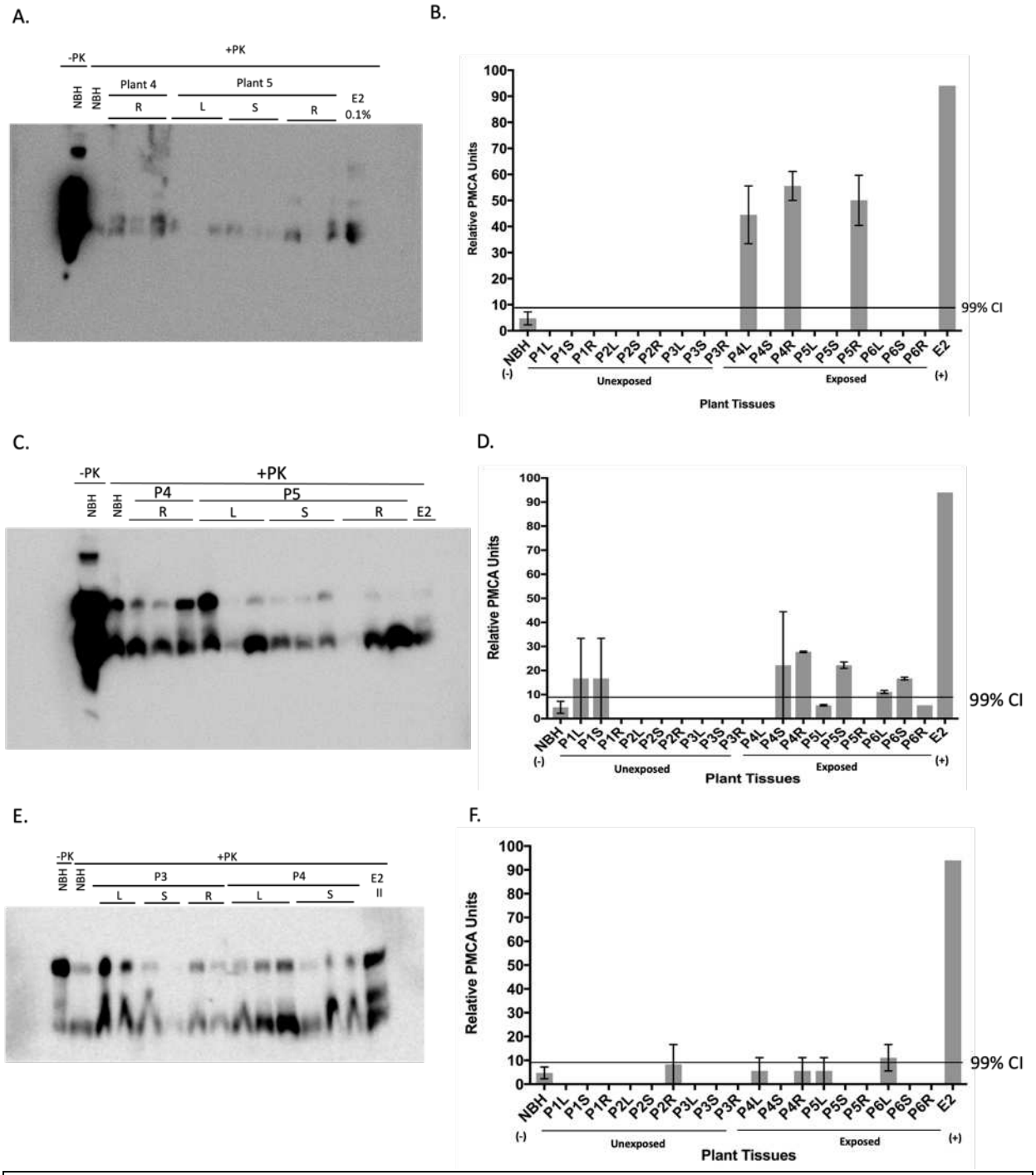

Figure 5.5. Prion positivity is detected in plant tissues at 2 h and 24 h post exposure, but not 72 h post exposure. Representative western blots after PMCA amplification are shown for each time point of 2 h (A), 24 h (C) and 72 h (E) post-exposure. Relative PMCA unit graphs (calculated as shown in Figure 5.4) are also shown for 2 h (B), 24 h (D), and 72 h (F) post exposure. 99% confidence intervals were calculated by taking the mean score of the spontaneous conversion of NBH negative controls plus four times the standard error of the mean. 99.9% CI are show on the graph. Based on the detection of E2 conversion, it was determined that the specificity of this experiment was 94%. Bars show the mean and standard error of the mean (SEM).

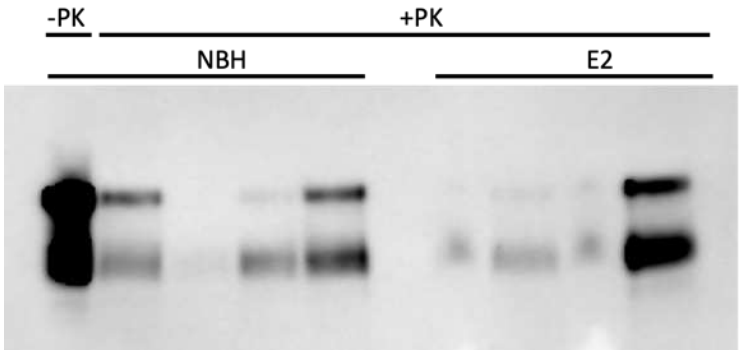

Figure 5.6. PMCA gave equivocal results. After successful experiments that were interpretable and quantifiable resulting in the data and conclusions above, PMCA became a major hurdle to completing and expanding on the data shown above. No electrophoretic differences were observed between E2 and NBH, both of which looked electrophoretically similar to undigested NBH.

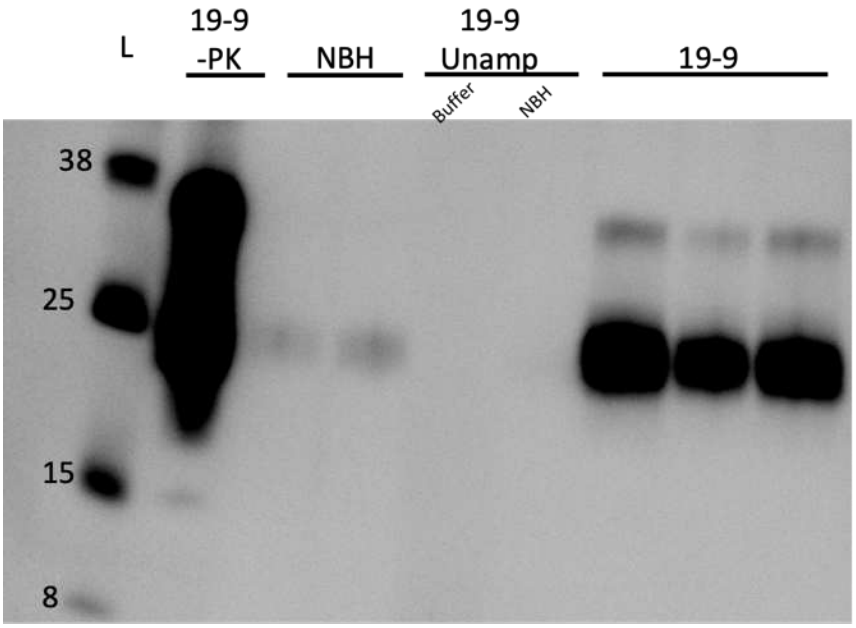

Figure 5.7. PMCA successfully amplified CWD prions from a white-tailed deer brain. After much troubleshooting, PMCA was attempted again on CWD prions from a white-tailed deer (19-9) using 1536 NBH that was gifted to us. The combination of protocol changes and NBH was likely able to ameliorate the issues that were being observed in PMCA. All samples were PK digested except where noted. Unamplified 19-9 was run as a PK digestion control to ensure that PK was working and the seed that was used in the assay was below the limit of detection before amplification. While amplified NBH was unable to be completely digested away, there is still a clear difference between amplified NBH negative controls and 19-9 positive samples. 19-9 was diluted both in buffer and NBH as an unamplified control. Unamp = unamplified, L = ladder. Numbers on the later show kDa.

Discussion

Chronic wasting disease is a devastating disease infecting free-ranging and captive cervid populations. The extraordinary transmissibility of CWD makes the disease nearly impossible to control once a herd is infected. In fact, some mathematical models predict that CWD will ultimately lead to cervid extinction events[25]. While the outcome of CWD infection in cervid populations is bleak, there are glimmers of hope. New York, for example, was able to identify, contain and prevent the spread of CWD after it was first identified in the state in 2005[37]. To increase the likelihood of more future successes with controlling CWD, every facet of disease transmission needs to be understood to better prevent, predict and mitigate future outbreaks.

Plants comprise the majority of the diets for cervid populations[31]. If plants themselves are either contaminated on the surface from an animal depositing prions into the environment or by taking up prions from contaminated soil, it is important to assess the role of plants in disease transmission. But can plants even uptake prions? It was often thought that plants required nitrogen-fixing bacteria or other symbiotic associations to acquire nitrogen, research has indicated that plants are able to consume nitrogen without the help of any symbiotic bacteria, indicating that the plants are able to extract the nitrogen from proteins they are exposed to in the soil[38]. The same study also found that intact proteins could also be taken up by the plant, likely by endocytosis, within the root system[38]. In fact, additional research demonstrated that wheat plants that secreted proteases from the roots were able to uptake nitrogen from protein alone[39]. Wheat plants have also been shown to uptake ovalbumin through their root system upon damage[40]. These data suggest that plants do have some intrinsic ability to uptake small proteins and amino acids and highlight the feasibility of plants being able to uptake prions.

While it does appear that plants have an intrinsic ability to uptake proteins, prions have been shown to tightly bind to soil and soil components. It is unknown how, or even if, the prions would become unbound to the soil and taken up by the plant. The world, however, is vast and complex. Perhaps there are fungal species that exist in soil that are able to disrupt the interactions between the soil and the prion enabling it to be taken up by the plant. Or, perhaps, the proteases secreted by the apoplast and root tissues of plants are sufficient to disrupt the interactions between the soil and the prion. For the purposes

of this study, however, it was decided to eliminate the confounding soil factor as much as possible to determine if rice plants have the ability to uptake infectious CWD prions.

The goal of this research was to better understand how/if plants contribute to the spread of CWD. To avoid the soil confounding factor, plants were intended to be acquired having been grown hydroponically, but the plants that were gifted to us were grown in soil. To eliminate as much of the soil factor as possible, plants were removed from the trays, the soil was removed and plants were gently washed with DI water. While every care was taken to not break/damage the root structures, there was some inevitable damage that occurred. Thus, it should be assumed that all the plants in this study had damaged root systems. Plants were then grown for the allotted time points before further analysis. In this particular study, food dye was also incorporated into the water and water + E2 that was given to the plants as a control that the plants were still taking in water as expected. Plant number 2 for all of these studies was exposed to only food dye to ensure that wouldn't be an issue in PMCA. While there wasn't a higher rate of spontaneous conversion in any of the plants designated as plant #2, it did clearly cause significant damage to the plant. For all samples, there were prions in the water given to plants that were supposed to receive prions, and there were no prions in the plants that were not exposed to prions (Figure 5.2). Of note, the prions in the plant fluid appeared to have lower signal that the controls run alongside that are either 10-fold or 100-fold more dilute. This suggested that the prions were either being taken up by the roots or degraded by the plants.

Unlike Rasmussen *et al*., we did not find any prions associated with the root tissue that was detectable before PMCA[35]. In fact, there was no prion positivity detected in any of our samples before amplification assays were employed (Figure 5.3). We were, however, able to detect amplifiable prions in the root tissue of 2/3 exposed plants at 2 hours post-exposure (hpe) and in the leaf tissues in 1/3 plants. One of the plants that had a positive root sample also have a positive leaf sample (Figure 5.4). These initial results suggested that plants were strongly associated with or taken internally by the root structures of 2/3 plant and for at least one of those plants, the positivity was in the leaf structures as well. It's unclear why the stem was not also positive in that plant, but perhaps proteins move quickly to peripheral tissues in this setting.

By 24 hours post exposure (hpe), there was more positivity noted in plant tissues: 1/3 roots, 2/3 stems and 1/3 stems had positivity that rose above the 99.9% confidence interval. These data suggest that the prions have been taken up by the plants and are being trafficked aerially from the root tissues. For the purposes of this experiment, data were semi-quantified in an attempt to control for spontaneous conversion resulting in false positives, as well as look at the relative load of prions between the plant tissues. While a number of samples (mostly from the 24 h timepoint) did not cross the 99% confidence interval that was used, there was some low level of amplification. Further studies using a larger sample size a repeating the data will provide insight if that signal is real. Overall, these data are suggesting that by 24 hpe, there is prion positivity in some root and some aerial tissues from exposed plants.

By 72 hpe, however, there are no amplifiable prions detected in any of the plant tissues. This could have happened for a number of reasons. The plants were no longer looking as healthy as when they were first exposed and perhaps as the plants were dying the prion signal was also diminished. Additionally, perhaps plants have an intrinsic ability to degrade the infectious prions, much like the fungal component of lichens[41]. This is not in agreement with the work shown by Pritzkow *et al*., but those experiments were conducted using a different type of plant (grass plants) and a different prion strain[36]. Maybe what we are seeing a plant-mediated degradation of the prions that results in the protein being unable to seed a PMCA reaction. Future studies that incorporate a 48 h time point and testing additional prion strains and/or plant species would help answer this question. Overall, these data provide more evidence to the growing body of work that suggest that plants are able to uptake prions through their root systems into their aerial structures and remain infectious.

Future experiments have been set up to expand upon the research presented here. Unfortunately, because of the technical difficulties surrounding the use of our amplification assays, these experiments have not been completed. The experiments that were attempted are described here. Rice plants were obtained as before (in soil), removed from soil and exposed to either Tg5037 NBH, prion-negative elk brain homogenate, or 1% E2 as before. For this set of experiments, no food dye was included for the reasons discussed earlier. These plants had 5 plants/group and were exposed for either 2, 24, 48 or 72h before processing as before. In addition to a repeat and expansion of the plant exposures, some rice plants were kept in their trays and exposed to 10 mL of 1% E2 or 1% NBH before

harvesting and processing. The soil was also saved to see if any prions could be detected either by electrophoresis, dialyzing the sample[42] or BASICs[43]. These experiments were designed to assess additional questions posed by the original experiment.

While these experiments were being set up, significant problems with PMCA began to arise (Figure 5.6), that were unable to be resolved (Table A.1). It wasn't until a few years later that some moderate PMCA success was again observed (Figure 5.7). Unfortunately, these experiments were unable to be tested by PMCA to date. Building upon the success of PMCA with 1536 NBH, future work will be aimed completing the experiments. Results from these experiments will provide additional insight into the potential role of plants in the transmission of CWD. Additional work to examine plants that were naturally exposed to CWD prions in Rocky Mountain National Park (RMNP) and from Arkansas for the presence of prions externally and internally were also met with the same technical difficulties. Future work with optimized PMCA protocols will be critical to provide evidence for CWD prions associated with plants in a natural environment. These technical challenges posed by PMCA were primarily observed when running PMCA using NBH from mice that overexpress cervid PrP, specifically the 5037 mice. While this is very frustrating for the purposes of PMCA, these results could provide insight into the unique characteristics of cervid PrP.

In summary, this work provides further evidence that plants may be able to be a short-term vector for CWD transmission. Specifically, the presence of infectious prions in plant tissues 24 h after exposure are alarming and may provide just another avenue for cervids to become exposed/infected. Future work analyzing the additional rice plant and soil experiments, as well as analyzing plants collected from areas where infected animals have congregated (from RMNP and Arkansas) will provide additional critical insights into the natural occurrence of this transmission. Additional studies using radioactively labeled prions would be interesting and insightful to determine how the prions are transported to aerial structures.

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Appendix

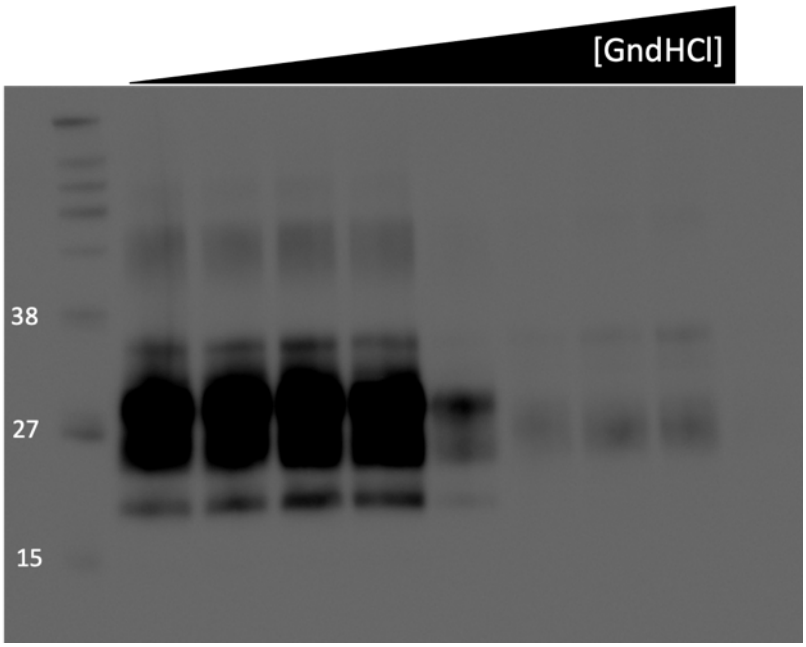

Figure A.1. E2 refolds into a PrP^C-like conformation after denaturation in ≥ 2.5 M GndHCl. E2 is a reference sample often used in our lab that is a full brain homogenate from a captive elk. When E2 was assessed for conformational stability, incubation in ≥2.5 M GndHCl resulted in a conformational change that gave a PrP^C-like electrophoretic shift, unlike what was seen in the white-tailed deer obex samples in Figure 2.1. These results demonstrate conformational differences between elk and white-tailed deer, brain-derived prions upon renaturation after treatment with GndHCl. Perhaps this is a result of polymorphisms at codon 226 between elk and white-tailed deer. The white star denotes 2.5 M GndHCl.

Table A.1. Experiments to troubleshoot PMCA.

Experiment Troubleshooting	Rational	Success?
PK Digest		
PK Digestion with SDS (50µg/mL PK, 0.02% SDS) for 30m	SDS can facilitate PK digestion and may help discriminate between E2 and NBH	No
PK Digestion in 50µg/mL for 1h instead of 30min	Longer time may be necessary for sufficient digestion	No
PK Digestion with SDS (50µg/mL PK, 0.02% SDS) for 1h	Longer time and the addition of a detergent may help with the PK digestion	No
PK Digestion with SDS (50µg/mL PK, 0.02% SDS) for 40m	Longer time and the addition of a detergent may help with the PK digestion, maybe won't	No
PK Digestion with Pre-Incubation with 0.2% SDS @ 37°C for 10min	Adding SDS ahead of the PK digestion may help break up prion aggregates	No
PK Digestion with Pre-Incubation @ 37°C for 10min (no SDS)	Does prewarming the sample make the intermediate band worse?	No
PK Digestion with Pre-Incubation @ 95°C for 10min (no SDS)	Perhaps a high temperature pretreatment will break apart the prion aggregate	No
PK Digestion with Pre-Incubation with 0.2% SDS @ 95°C for 10min	Will higher heat + detergent help break the intermediate apart?	No
PK Digestion with 100µg/mL PK, 0.2% SDS for 30m @ 37°C	Will a higher concentration of PK and the addition of SDS help with digesting the intermediate band?	No
PK Digestion Protocol - 50µg/mL PK, 1% SDS, 0.025% Triton-X, 37°C for 40min	Will following the PK digestion protocol from the H/M lab help with digesting the intermediate band?	No
PK Digestion adding 0.5M EDTA back into the reaction	In Pulford et al. (2012), there is EDTA in the digestion reaction and perhaps it will help with eliminating the intermediate band signal	No
NBH		
Test using NBH gifted from Telling lab	If there is something wrong with our reagents/weird with our mice, perhaps another lab's NBH will help the problems we've been having	No
Clarified/unclarified NBH	A slow spin (1,000 rpm for 1 min) will help separate the NBH sample and will maybe make it easier to work with. This was suggested in a lab meeting	No
Making NBH with mouse brains from Telling/Mathiason labs	If there is something weird happening with our mice, using mice from another colony might fix the issue	No
Different batch of NBH	Will NBH prepped at a different time give different results?	No
Different researcher made NBH	Perhaps someone else will have better luck prepping NBH/be better at perfusing the mice	No
Telling lab brains prepped using Telling protocol	Maybe a different protocol will give us better results	No
Protocol changes in sonication time/intensity/PMCA beads		
Telling protocol (different sonication settings, addition of PMCA beads, smaller sample volume, different tubes) – did multiple experiments with all these interations	Will using a different protocol from another lab that has PMCA success help us also be successful?	No
Telling sonicator with Telling protocol and Zabel protocol	If there is something wrong with the Zabel sonicator, we should see success using the Telling protocol and see a difference between the protocols	No
Verifying temperature stability in sonicator	Is the temperature consistant and in an acceptable range for prion amplification	No
Different scientist set up PMCA	Can someone else get this to work? Is it just me?	No
Equipment		
Resurfaced sonicator horns/whole new sonicator	Perhaps the equipment is faulty and repairing/replacing will fix the issue	No
Used both Telling and Zabel sonicators	Perhaps one works better than the other	No
When does this "intermediate band" show up? Can we use this information to help decide how to prevent it?		
Freeze/thaw?	Is a simple freeze thaw enough to create this troublesome band? (Yes)	No
37°C for 1h?	We wanted to see if a simple incubation at 37°C incubation would result in the presence of this signal (yes)	No
Iterations of clarified/unclarified NBH, Zabel or Telling mouse brains, Zabel or Telling protocol	Is there a combination of factors that will influence the intensity/frequency of this intermediate band presence (no)	No
Can we differentiate between NBH and E2 at any frequency using Zabel methods?	If I set up 40 NBH and E2 samples over a few experiments, will we be able to differentiate between the positive and negative samples? (No, 40/40 looked the same and were unable to be differentiated)	No