DISSERTATION

PRIONS IN THE ENVIRONMENT: FROM THE HOST TO THE ENVIRONMENT AND BACK AGAIN

Submitted by

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ABSTRACT

PRIONS IN THE ENVIRONMENT: FROM THE HOST TO THE ENVIRONMENT AND BACK AGAIN

Prions, misfolded isoforms of normal mammalian prion protein, are the putative infectious agents of transmissible spongiform encephalopathies (TSE). Prions are implicated in neurodegenerative diseases such as Kuru, Creutzfeldt-Jakob disease (CJD), variant CJD (vCJD), chronic wasting disease (CWD) in cervids, bovine spongiform encephalopathy (BSE) in cattle, and transmissible mink encephalopathy in mink. The unusual nature of prions, an infectious protein, has challenged the concept of transmissible pathogens and created a formidable challenge for detection and study of the agent.

To date, CWD is the only naturally occurring TSE of free-ranging animals. As compared to other TSEs, CWD appears to be highly transmissible and very robust to degradation. Studies suggest that environmental deposits of chronic wasting disease prions (PrP^{RES}) play an important role in the transmission and persistence of CWD among captive and wild cervids. Furthermore, studies indicate that prions form a close association with clays and other soil components, enhancing their persistence and surprisingly, enhancing the transmissibility of the infectious agent. Variability in apparent infectious titers of prions when bound to soil has complicated attempts to quantify the binding capacity of soil for prion infectivity.

In the first chapter of this dissertation we examine the binding capacity of prions to soil by a subtractive infectivity bioassay. We quantified the prion adsorption capacity of whole, sandy loam soil (SLS) typically found in CWD-endemic areas in Colorado; and purified montmorillonite clay (Mte), previously shown to bind prions, by BioAssay of Subtracted

Infectivity in Complex Solutions (BASICS). For 24 hours we incubated prion positive 10% brain homogenate from terminally sick mice infected with the Rocky Mountain Lab strain of mouse-adapted prions (RML) with 10% SLS or Mte. Samples were centrifuged and soil-free supernatant was intracerebrally inoculated into prion susceptible mice. We used the number of days post inoculation to clinical disease to calculate the infectious titer remaining in the supernatant, which we subtracted from the starting titer to determine the infectious prion binding capacity of SLS and Mte. BASICS indicated SLS bound and removed 95% of infectivity. Mte bound and removed lethal doses (99.98%) of prions from inocula suspension, effectively preventing disease in the mice. These data reveal significant prion-binding capacity of soil and the utility of BASICS to estimate prion loads and investigate persistence and decomposition in the environment. Additionally, since Mte successfully rescued the mice from prion disease, Mte might be used for remediation and decontamination protocols.

Currently, few detection, and no quantification methods exist for prions naturally deposited in soil, hindering an understanding of prion persistence and infectivity in the environment. Serial protein misfolding cyclic amplification (sPMCA) currently has the greatest prion amplification capabilities but unfortunately has had limited success in detecting prions in soil due to unidentified inhibition and the tight adsorption of the prion with soil components. In the second chapter of this dissertation we outline efforts to develop a novel detection assay using aptamers, small oligonucleotieds capable of epitope specific labeling. Our objective was to develop this assay to allow for an increased detection limit of prions in soil and tissue samples. In collaboration with InfoScitex we selected for aptamers reactive against PrP^{RES}, candidate aptamers were purified and incubated with positive and negative control samples. PrP^{RES} detection by western blot showed some promise for two prion strains but was difficult to

replicate and did not work for CWD. A second method of amplification was tested for samples containing prions that were below the detection threshold of a western blot. Bound aptamers would then be amplified by rtPCR. Data from this method was inconsistent and indicated a non-specific binding to negative elk brain homogenate molecules. Further aptamer selection and research should be pursued with this technique to achieve targeted PrP^{RES} binding.

Enhanced surveillance of CWD in both free-ranging and captive elk and deer herds is an essential aspect of study and management of this disease. Currently, immunohistochemistry (IHC) is the gold standard for CWD diagnosis in cervids. In the third chapter of this dissertation we compare the sensitivity and specificity of IHC to sPMCA. Obex samples from free-ranging Rocky Mountain elk were blindly tested by sPMCA and compared to IHC findings of PrP^{RES} in obex and lymph nodes of the same animals. Hierarchical Bayesian analysis found the sensitivity of sPMCA on obex tissue (95%) was higher than IHC on the same obex tissue (71%). Only when IHC was used on three different tissues did the sensitivity match that of sPMCA. These data are significant for the identification of a previously unrecognized sub-clinical population on the landscape, potentially capable of shedding and transmitting CWD. Additionally, our findings challenge the idea of CWD being an invariably fatal disease, instead suggesting a possibly infectious but subclinical or carrier state may be more common than previously believed.

The transmission ecology and epidemiology of CWD through environmental reservoirs is poorly understood due to the novelty of the agent and the difficulty of prion detection in environmental samples. It is unclear what an infectious dose is in a natural setting, and if a difference in transmission rate between a single or chronic exposure exists. In the fourth chapter of this dissertation we test chronic exposure to CWD in naturally contaminated soils. Our objectives were to investigate the role of indirect transmission by exposing PrP^{CWD} susceptible

transgenic mice to PrP^{CWD} contaminated soil, to evaluate a dose response of prions in soil, to estimate average soil ingestion by laboratory mice, and to estimate annual exposure to prions from chronic environmental exposure of prion contaminated soil. This study was the first to successfully use transgenic mice to test soil infectivity from natural sources. We found that chronic exposure was more efficient for CWD transmission than a single concentrated oral dose of CWD-spiked soil. Epidemiology of the studies also suggests that infectivity differences existed between our two sources of naturally contaminated soil, it is unclear if a difference in titer or CWD strain is responsible. Additionally through soil ingestion estimates we calculated an annual ingestion of $> 3.6x10^5$ LD_{50} infectious units by our mouse model.

Taken together we show that soil is capable of acting as a reservoir by binding infectious prions that are still biologically available to infect hosts upon ingestion. Our research demonstrates the importance and sensitivity of PMCA as a diagnostic tool for detecting pre-clinical infections in elk and mouse models. Understanding the relationship between CWD prions, the host species and the environment requires better detection methods, estimates of prions in the environment and data on the behavior of soil and prions during transmission of CWD.

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DEDICATION

I would like to dedicate this work to my family and friends, who have supported, cajoled, teased, reinforced and believe in me. Despite many of their fears, I intend for this to be the completion of my life as a registered student, but certainly not the end of being a student of life. Thank you each for your faith in me, it has meant everything.

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INTRODUCTION

Prions and their History

Prions are the etiological agent of neurological diseases termed transmissible spongiform encephalopathies (TSE). Scrapie, the first recognized TSE, was documented as affecting sheep and goats as early as the 1700s [1-10]. In the 1920s medical doctors recognized a similar neurodegenerative disease in humans which was later named Creutzfeldt-Jakob Disease (CJD) after the discovering doctors, Hans Creutzfeldt and Alfons Jakob [11-13]. Later, in the 1960s, Kuru, a devastating disease of the Papua New Guinea Fore people, was also identified as a TSE, with an epidemiology suggesting transmission through cannibalistic traditions [14,15].

Experimental and anecdotal evidence indicated that both scrapie and Kuru were transmissible diseases [3,8,9,15-18], however, laboratory techniques available at the time could not identify the disease agent. The agent was resistant to UV light, high temperatures and high pressures, thereby ruling out known bacteria and viruses, and challenging the research community to identify a new category of agent [6,19,20]. As a result of this and the slow disease course, it was hypothesized that TSE's were caused by a new type of "slow virus" that was too small to purify but had virus like phenotypes such as transmissibility and heritability [5,19]. This was the predominantly held theory until 1982, when Stanley Prusiner [21,22] proposed that the causative agent was exclusively a protein. He solidified the "protein-only" hypothesis which had been developing and termed the agent ***pr***oteinacious ***i***nfectious ***on***ly, or prion [1,19,23].

The idea of an infectious protein that exhibited heritable traits violated the dogma of molecular biology in which infectious organisms by definition included instructional genetic

code, in the form of DNA or RNA. The presence of this instructional material in bacteria and viruses allowed for propagation, phenotypic inheritance of strains and evolution of phenotypes. Scrapie prions had shown phenotypic changes associated with crossing species barriers and had repeatedly produced different and reproducible variants, or strains, in experimental systems [21,24-27]. Both of these characteristics were typical observations in virology, and were difficult to explain with a protein-only pathogen [1,8,27-29]. As a result, it took two more decades for the protein-only hypothesis to be convincingly supported through biochemical and molecular studies.

Today prions are recognized as misfolded, pathologic isoforms of the normal, and endogenously expressed, mammalian prion protein (PrP^{C}), which can uniquely cause infectious, inherited or spontaneous disease. Prions are implicated in a number of human diseases such as Creutzfeldt-Jakob disease (CJD) [30], variant CJD (vCJD) [27], Kuru [31], Fatal Familial Insomnia [32] and Gerstmann Straussler-Scheinker disease (GSS) [33]. Animal prion diseases include chronic wasting disease (CWD) in cervids [34], scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle [35], and transmissible mink encephalopathy (TME) in mink [36].

Clinical manifestations of prion diseases generally include progressive neurologic deterioration resulting in ataxia, dementia, and behavioral changes. The diagnostic hallmark of a TSE disease is the presence of extracellular plaques composed of prion aggregates in neurologic or lymphatic tissues [17,34,37,38]. Generally, prion plaque deposits are associated with spongiform destruction to brain tissue and elevated levels of astrogliosis in the CNS.

The presence of neural astrogliosis is one of the few indicators of an immune response in the host, which is not surprising since PrP^{C} is an endogenous host molecule that the immune

system recognizes as self. Immune tolerance to the identically sequenced PrP^{RES} prevents an antibody response in competent hosts. Only in PrP knock out or null (PrP%) mice is it possible to stimulate an anti-PrP antibody production with a prion vaccine [39]. Curiously, evidence exists of increased germinal centers in the lymph nodes of infected animals. In fact, most immunological evidence suggests that the prion evades host recognition and instead utilizes the host immune cells to infect the CNS. Studies have implicated follicular dendritic cells (FDCs), B lymphocytes, phagocytes, the complement pathway and the gut associated lymphatic tissue (GALT) in peripheral prion infection and replication [40-43].

Though there are examples of prion transmission through iatrogenic means or infection through blood or tissue grafts, the primary and most natural route is through oral-nasal exposure. This is true for Kuru, BSE, vCJD, TME, scrapie and CWD [15,30,44-47]. Upon ingestion, studies suggest that the prion infects the GALT, which includes M cells, Peyer's patches (PP) and the follicle-associated epithelium (FAE). In challenge studies the PPs appeared to be the first infected tissue, with detectable prions associated with lymphatic tissue in as early as one week of oral inoculation [47-50]. M cells sample the prions from the intestinal lumen then traffic the prion to the PP [51,52]. Once inside the PP the prion is trafficked by macrophages and DCs to germinal centers in lymph tissues including mesenteric lymph node, retropharyngeal lymph node and spleens, where they are transferred to FDCs [52-55]. Studies suggest that the process of prion replication and retrograde neuroinvasion is dependent FDCs and their proximity to the enteric nervous system [56,57].

The general nomenclature for the infectious agent is PrP^{Sc}, for the pathologic isoform associated with scrapie, or generically PrP^{RES} since the isoform is generally resistant to protease degradation. Other biochemical hallmarks include conversion of the normal α helical rich PrP^{C}

conformation to a β sheet rich PrP^{RES} form. This conformational change allows for the formation of insoluble amyloidogenic aggregates [58]. The exact tertiary structure of the prion protein in either conformation is not entirely clear since both forms form contains a flexibly disordered N terminus precluding the effective use of x-ray crystallography. Additionally, the misfolded form, PrP^{RES}, occurs in insoluble aggregates preventing monomer analysis.

Sequencing, circular dichroism (CD) and nuclear magnetic resonance (NMR) suggest a few common features across prion diseases despite differences in host prion sequences. The prion protein is a glycosyl phosphatidyl inositol-linked glycoprotein anchored in cholesterol and sphingolipid-rich rafts of the cell membrane. Differential glycosylation at N181 and N197 produce di-, mono- or un-glycosolated forms ranging in molecular weight from approximately 27-38 Kd. Analysis by CD found that PrP^{C} is ~42% alpha helical with only 3% beta sheets, but the misfolded isoform, PrP^{Sc} is ~30% alpha helical with 43-54% beta sheet richness [59]. The disordered N terminal tail contains octapeptide repeats capable of binding divalent cations such as Cu^{2+} and Mn^{2+} [60]. The 19-30 Kd PK resistant core of PrP^{RES} contains a large loop located between the second β-strand and second α-helix [61-64]. Analysis of recombinant proteins by NMR shows this loop to be extremely flexible in most species, including humans, bovine (*Box spp.*), sheep (*Ovis aries)*, mouse (*Mus musculus*), and hamsters (*Mesocricetus auratus*), but it is almost entirely rigid in the prion protein of (*Cervus elaphus*) and deer (*Odocoileus spp*.) [63-67]. The significance of this loop is not known, though it may play a role in the observed species barrier of CWD [68-70]. Overall, sequence analyses the prion gene (Prnp) show the prion protein to be highly conserved across the mammalian class. This conservation suggests a role in homeostasis and the maintenance of the protein through selection pressures [71-74].

Despite apparent evolutionary conservation the primary role of PrP^{C} is unclear. The protein is found in nearly every tissue including heart [75], skeletal muscle [76,77], kidney [75,78] and most extensively central nervous system and lymphatic cells and tissues [75,79,80]. PrP^{C} appears to be involved in a variety of biological pathways ranging from protective to pro-apoptotic [81]. It has been implicated in functions as diverse as cell signaling [82], cell proliferation [83], immune modulation [84], copper binding [85], binding of Aβ in Alzheimer's disease [86,87] and both pro- and anti-apoptotic signaling [88,89]. Despite these roles the protein plays, genetically deficient prion protein ($PrP^{\%}$) mice are considered generally normal suggesting that the disease is not a result of loss of function pathology [83,90,91].

Prion detection and study

Today the protein-only hypothesis is widely accepted based on the experimental findings from both *in vivo* and *in vitro* studies. *In vivo* studies have extensively used both knock-out [92] and knock-in [93] forms of transgenic mice to aid the investigations of disease susceptibility, species barriers, disease pathogenesis and cellular processes [92-95].

$PrP^{\%}$ animals are completely refractory to infection [91,96], supporting the idea that the misfolded isoform, or PrP^{RES}, once formed requires PrP^{C} for autocatalytic propagation, recruiting and converting endogenous neighboring PrP^{C} molecules into new PrP^{RES} molecules. This recruitment and misfolding results in the formation of well-structured aggregates and fibrils, which form the histological plaques commonly associated with this disease. Conversely, Prnp over-expressing mice have demonstrated age-related spontaneous disease in the absences of other pathogens [97,98]. Prnp sequence substitutions and protein manipulations have created model animals allowing for prion disease studies looking at genotypic effects, impact of sequence shuffling and behavior of truncated peptides [95,99-102]. Species barriers are

characterized by the ability of a prion strain to infect a novel host. The extent of species barriers were, and continue to be, tested by creating and experimenting on mice expressing the Prnp gene of a variety of species including elk, deer, human, sheep and even horses [69,103-106]. A species refractory to the inoculation demonstrates a species barrier.

In vitro studies allowed for biochemical analysis of PrP^{C} conformational stability, differences between strains [103], as well as the behavioral differences of the native protein compared to recombinant prions [10,107]. Aspects of the species barrier were tested and manipulated using a variety of laboratory assays [69,108]. *In vitro* techniques allowed researchers to model replication events and manipulate components and conditions to identify what is necessary for protein misfolding [107,109,110].

The most common application of *in vitro* methods is detection and diagnostics of prion diseases [111-115]. Methods include immune-detection assays such as western blots, enzyme linked immunosorbant assays (ELISA) and immunohistochemistry (IHC) which provide direct detection of the prion protein in both the normal and misfolded form [6,114,116-118]. IHC is currently considered the diagnostic gold standard and provides direct visualization of the prion amyloid deposits present in tissues. IHC is instrumental in mapping prion distribution throughout tissues, as well as differentiating between prion phenotypes such as punctate verse diffuse amyloid staining. Western blots and ELISAs also use antibodies to directly detect the presence of prions in a liquid sample and are commonly used for disease diagnostics.

Serial protein misfolding amplification assay (sPMCA) was the first *in vitro* amplifying assay developed for prions. It is extensively used for detection of prions in many tissues of many species [119-123]. sPMCA is also an integral tool in testing the conditions and components that lead to protein misfolding and the infectious state across all strains of prions [109,124].

Analogous to PCR, sPMCA provides a rapid and sensitive method of prion amplification and detection in a relatively short period of 1-2 weeks. The sample of question, such as homogenized brain tissue from a suspected positive animal, acts as a prion template. This sample is combined with uninfected normal brain homogenate from transgenic mice expressing a surplus of PrP^{C}, which acts as substrate. Repeated incubation and sonication cycles achieve sufficient amplification of PrP^{RES} for detection by immunoblot. Most sample types are successfully tested by sPMCA, however, this assay has limited success amplifying prions in blood, urine, feces, and environmental samples. An inhibitory effect seems to be in play, but is yet to be identified.

Real-time quaking-induced conversion (RT-QuIC) provides an additional amplification assay with similar sensitivity to sPMCA [125,126]. RT-QuIC, unlike other *in vitro* prion assays does not use antibodies for detection. Instead, prion amplification is measured by Thioflavin T, a dye capable of emitting a fluorescent signal when intercolated into aggregated proteins. The increasing production of aggregates during amplification is measured in real-time as the Thioflavin T fluorescence increases. Interestingly unlike sPMCA, RT-QuIC does not create an infectious product, and recombinant hamster PrP^{C} appears to be a ubiquitous substrate in this assay. The reasons for these unusual observations are under investigation.

The most significant goal thus far achieved with *in vitro* experiments has been the validation of the protein-only hypothesis. Two different studies have been able to form *de novo* infectious prions from minimal synthetic components. Zhang et. al. [127] used bacterially expressed recombinant PrP (rPrP) to create *de novo* infectious prions in a prion free sPMCA experiment. The ability of rPrP to misfold, become PK resistant and be infectious is the strongest evidence that the protein-only hypothesis is true. This is further supported by work from Supattapone et. al. [124], which has shown that purified, native form PrP^{C} can be converted *de*

novo to a misfolded and infectious form of PrP^{RES} through sPMCA with the introduction of non-proteinaceous co-factors. Through experimental elimination it has been found that both non-specific RNA and phospholipids are required for production of misfolded infectious PrP^{RES} [109,128-130]. The exact mechanisms of these, and possibly other co-factors, as catalysts or otherwise essential components is under further investigation [124].

Prion Disease Pathology and Strains

It is still unclear if disease pathology is a result of fibril toxicity or if the deposition of protein plaques in the neurological tissue is a by-product of disease [131]. What is known is that sufficient PrP^{C} is required for disease progression. This is proposed as a leverage point in treating the disease by reducing the amount of ambient PrP^{C} present in the host, thereby slowing or stopping disease progression [132,133]. Therapies utilizing this strategy and others are underway with siRNA to knock down PrP^{C} production, or vaccine induced anti-PrP antibodies to sequester and destroy endogenous PrP^{C} [39,134-136].

A remaining contentious issue in the prion hypothesis is the observation of strains or variants of prions. Through biological cloning, scrapie was first observed to differentiate into distinct strains in 1969 [137]. These strains were distinguished by days to clinical disease after inoculation (days post infection (dpi)), clinical signs in bioassay animals, and pathology upon microscopic analysis of prion deposits. Today it is believed that both strains and isotypes occur in nearly all prion diseases [46,94,138,139]. For example, CWD-positive brain samples from different elk are considered separate isolates. Upon passage into another elk or into susceptible mice, differences might be observed in average dpi or in the type or severity of different clinical signs. Upon post-mortem examination different distribution patterns in brain or peripheral tissues might be observed, intensity and appearance of prion deposits and spongiosis might differ, or

biochemical aspects of the prion itself such as glycoform ratio or PK resistance may vary. If these aspects are heritable upon sequential bioassay passage, it would generally be considered a strain [140].

An example of the strain phenomenon would be the Hyper and Drowsy Syrian hamster scrapie strains [79,141]. These two experimental scrapie strains were biologically cloned from the same Syrian hamster strain, but upon serial passage they became very distinct and extremely reproducible. The differences in these two strains were used to look at co-infection and the resulting competition, or inhibition, of one strain by another [142,143] exemplifying the existence of distinct strains.

The question remains, if both Hyper and Drowsy have identical amino acid sequences, how can they result in different heritable phenotypes? The prevailing hypothesis is that differences in protein conformation directly influence phenotypes, and that those conformations are transmissible, and as such heritable. This theory is supported by extensive research including a study by Telling et. al. [102], which inoculated FFI and two variants of CJD prions, familial and sporadic, into susceptible mice. The resulting PK resistant prion fragments recovered from mouse brains were identical in molecular weight to the agent of inoculation. A second study by Colby et. al. [144] found that mice inoculated with aggregated recombinant proteins of differing conformational stabilities contracted disease from those proteins. The material isolated from these mouse brains showed host produced prions with the identical conformational stability as the respective inoculums.

The idea of prion strains raises the question of evolution and adaptation of the prion as a disease agent. Without genetic code, this idea has been hotly contested, however, there is mounting evidence that prions are subject to natural selection just like any other pathogen. Li et.

al. [145] demonstrated that the use of swainsonine, an agent that impairs formation of complex N-linked glycans, could create a selection pressure sufficient to drive the emergence of different mutants within a previously homogenous population. Similar selection pressure has been shown in the host environment, with prion strains adapting to as yet unidentified host factors both *in vitro* and *in vivo* [103,146]. These studies demonstrate the plasticity of the prion to adapt and replicate in the host environment. Findings of this type highlight the unusual nature of prions as a disease agent and fuel further research to better define this pathogen.

Prions, and their resulting TSE diseases, were not commonly known to the general public until the occurrence of BSE and the epidemiologically linking it to vCJD. The possibility of prion transmission to humans spurred a large research effort into BSE, vCJD, CJD, scrapie, CWD and other prion diseases. This extensive research on prions as a disease agent has led to the recognized commonalities between prion diseases and other protein misfolding diseases. The "prion like", or prionoid term is now applied to diseases that include, Alzheimer's, Parkinson's, ALS, Huntington's Disease, and many more [147,148]. While most recognized protein misfolding diseases are not considered transmissible at the host level, the cell to cell and tissue to tissue transmission that occurs in the host is very similar to prion misfolding, replication and transmission [149,150]. These diseases, similar to prion diseases, can result from inherited or acquired mutations, spontaneous misfolding events, or environmental or stress factors not yet identified. Today extensive collaboration and cross-pollination occur between the protein misfolding research fields.

CWD

CWD is a TSE first seen in captive Colorado and Wyoming cervid populations in 1967, and later found in free-ranging populations in 1981 [151]. In 1980 Williams and Young [34]

identified the disease as a TSE. Since it's identification in the wild 1980s CWD has been found in domestic and free-ranging populations in 22 US states and two Canadian provinces [152], and was unintentionally exported to South Korea through wildlife trade [153] (Table 1.1). Increased surveillance and relocated animals (Saskatchewan and South Korea) contributed to the ever-increasing range and prevalence of CWD. However, the continued range expansion of endemic areas of the disease is likely from natural transmission between animals at the landscape level [45,154-156].

Table 1.1. Summary of first documented incidence of chronic wasting disease around the world (modified from www.cwd-info.org CWD timeline)

Year	**Species**	**Captive/Free-ranging**	**State**
1967	Mule deer	Captive	Colorado
1979	Mule deer Black-tailed deer Elk	Captive Captive Captive	Wyoming Wyoming Wyoming
1981	Elk	Wild	Colorado
1985	Mule deer Mule deer	Wild Wild	Colorado Wyoming
1996	Elk	Captive	Saskatchewan
1997	Elk	Captive	South Dakota
1999	Elk Mule deer	Captive Wild	Montana Nebraska

	Elk	Captive	Oklahoma
2000	Mule deer	Wild	Saskatchewan
2001	White-tailed deer	Wild	South Dakota
	White-tailed deer	Captive	Nebraska
	Elk	Captive	South Korea
2002	White-tailed deer	Wild	Wisconsin
	Mule deer	Wild	New Mexico
	Elk	Captive	Minnesota
	White-tailed deer	Captive	Wisconsin
	White-tailed deer	Wild	Illinois
	Elk	Wild	South Dakota
	White-tailed deer	Captive	Alberta
2003	Mule deer	Wild	Utah
2005	White-tailed deer	Captive	New York
	White-tailed deer	Wild	New York
	White-tailed deer	Wild	West Virginia
	Moose	Wild	Colorado
	Mule deer	Wild	Alberta
	Elk	Wild	New Mexico
2006	White-tailed deer	Wild	Alberta
	White-tailed deer	Wild	Kansas
	White-tailed deer	Captive	Minnesota
	Mule deer	Wild	New Mexico

2008	Elk White-tailed deer Moose	Wild Captive Wild	Saskatchewan Michigan Wyoming
2010	White-tailed deer White-tailed deer Mule deer	Wild Captive Wild	Virginia Missouri North Dakota
2011	White-tailed deer White-tailed deer	Wild Wild	Maryland Minnesota
2012	White-tailed deer White-tailed deer White-tailed deer White-tailed deer	Captive Wild Captive Wild	Iowa Missouri Pennsylvania Texas
2013	White-tailed deer	Wild	Pennsylvania

It is unclear where or how CWD emerged in cervids, one theory is emergence from a cross-species transmission event from sheep grazing in the Colorado hills. A second origin theory is similar to the origin of scrapie, CWD may have been an endemic but extremely rare disease that eventually became established in a population. Conceivably it could have occurred in a similar fashion to spontaneous CJD in humans, which is known to affect 1 out of every 1 million individuals each year. A disease of that frequency in wildlife would be difficult to observe and identify, unless it occurred in a captive animal as was seen in the research facilities in Fort Collins in 1967. It is possible, though impossible to prove, that a single individual in captivity may have started the subsequent transmission and eventual population level spread of

CWD throughout North America [157]. Regardless of its origin, the increasing incidence and prevalence across North America continues, with new states reporting positives each year (Table 1.1).

CWD is the only known TSE to occur in free-ranging wildlife populations [151]. It has been found in elk (*Cervis elaphus*), mule deer (*Odocoileus hemionus),* white-tailed deer (*O. virginianus*), black-tailed deer (*Odocoileus hemionus columbianus*) and moose (*Alces alces shirasi*) [34,151,158]. CWD prions are highly transmissible and appear to be transmitted horizontally or indirectly from contaminated environments [159-163]. As compared to other prion diseases it is robustly resistant to environmental degradation [18,163,164] resulting in long-term persistence and accumulation in the environment. Prevalence of CWD ranges from <1% to 45% in free-ranging populations and has been documented as high as 90% in a captive herds [34,165-167]. Recent studies indicate that despite the slow disease course, the high prevalence in free-ranging populations will have long-term impacts on herd recruitment and population size [167].

CWD, Human Health and Management

In the wake of the BSE epidemic in Britain during the 1980s and 90s and the link to vCJD there was a sudden concern that CWD might also jump the species barrier and infect humans through the consumption of game meat. Although some state agencies were already collecting prevalence data from hunter-harvested samples, the effort was redoubled in CWD-infected herds, and nascent programs were started in adjacent states and states which had captive-cervid operations [168]. Additionally, translocation and wildlife feeding or baiting were banned in many areas with CWD. Early in the epidemic, wildlife managers, politicians and the public hoped that disease management or even eradication might work in new focal points.

Wildlife agencies tested aggressive approaches, delineating endemic zones, increasing game licenses sales, utilizing sharpshooters, as well as test and cull efforts [157,169,170]. Despite these extensive efforts, management strategies were unsuccessful in containing or reducing CWD prevalence in designated areas. Today, the idea of eradication has been abandoned, and even efforts to control CWD are called into question. The environmental contamination of prions likely contributes to the ever increasing prevalence, limiting the efficacy of population management strategies [171,172].

The public's concern about the risk of transmission to humans resulted in precipitous drops in hunting related sales [173,174] and an increase in demand for experimental and epidemiologic studies. Extensive testing and modeling of the cervid-human species barrier has been conducted both *in vitro* and *in vivo*. Sequence and conformation analysis, as well as template conversion experiments have shown differences of scrapie and CWD PrP^{RES} from human PrP^{C} which prevents templating and conversion [63,175,176]. Additionally, humanized transgenic mice have been inefficient in transmission [37,106,177]. As of yet, no epidemiologic evidence exists indicating CWD transmission to humans in communities that regularly consume wild game meat [178-180], however, long-term epidemiology studies continue [181,182] and the Center for Disease Control and Prevention (CDC) advises testing wild game and not eating CWD-positive venison.

CWD transmission, detection, pathogenesis and immune trafficking

Neuropathology of CWD includes neurodegeneration, presented as the classic TSE spongiform transformation of neural tissues, and PrP^{CWD} aggregates in brain tissue [34]. The degeneration of the neural tissues results in behavioral changes and clinical signs including

chronic weight loss, ataxia, rough hair coat, polydipsia, polyuria, excessive salivation, teeth grinding, hyperexcitability and eventual death [34].

For reasons that remain unclear, indirect environmental transmission of prions appears to be limited to scrapie and CWD prions, and does not appear to be an ecological component of BSE or other TSEs. This phenomenon may relate to scrapie and CWD sharing similar lymphotropic shedding and transmission characteristics [53,140,183]. Infectious prions are likely deposited into the environment through alimentary shedding [120,184], placental material [185], antler velvet [121] and the decomposition of prion-positive mortalities [161]. Once in the environment, PrP^{RES} adsorbs strongly to soil components [9,186,187], remains infectious [21,188,189] and persists for years [161,163,188,190]. Indirect transmission most likely occurs through incidental and geophagic ingestion of soil or other contaminated fomites, as well as deer sign-post behavior [160,161,191].

Experimental evidence suggests that the particularly strong adsorption of prions to soil colloids, or clays (defined as particles < 4 μm), may be responsible for their longevity in the environment [9,186]. With increasing clay content of soils increase in cation exchange capacity in their overall negative charge. Electrostatic and hydrophobic interactions between the prion protein and clay are thought to mediate this non-specific adsorption activity [8,10,28,192,193]. Specifically, montmorillonite (Mte), the most commonly occurring smectite clay, has been implicated in the adsorption of prions in the environment [3]. Mte is a 2:1 phyllosilicate clay consisting of 2 tetrahedral silica composed molecules flanking one octahedral aluminum composed molecule, forming a sheet. An interlayer space exists between sheets capable of expanding to > 2 nm depending on the cationic concentration of the solution. It is hypothesized that prions may enter this interlayer area like other proteins. However, Johnson et. al. [3] did not

find evidence of this in their experimental system and other studies suggest extensive protein unfolding would be required [194], which is unlikely for PrP^{RES}. Mte is prevalent throughout the US mountain west, including CWD-endemic areas [172,195]. Models suggest that the prevalence of Mte at a landscape level may explain and predict CWD prevalence, which can exceed 45% in free-ranging cervids [172,196].

Other soil components such as organic matter, quartz, minerals, tannins and humic acid have also been implicated in prion adsorption [1,3,5-10]. Whole soil commonly includes highly reactive humic substances, which have large specific surface areas and high binding capacities [11]. Humic substances can coat mineral surfaces imparting a net negative charge [14]. However, due to the unknown tertiary structure of PrP^{RES}, specific interactions and adsorption dynamics to soil and humic substrates have been difficult to verify.

Strong adsorption of prions in soils have proven difficult to measure or reverse, limiting prion detection sensitivity, estimation of prion adsorption capacity of soil [3,8,9,18] and general progress in studying prions in the environment. Additionally, prion detection in soil has been successful only in laboratory experiments using a variety of different methods including antibody labeling [6], electrophoresis [5], bioassay [21], detergent extraction [1] and protein misfolding cyclic amplification (PMCA) [21,25]. All of these studies to date have had low detection limits, which are not representative of the expected prion levels in nature.

To date, prion-soil interactions have largely been demonstrated with recombinant prion proteins [1,8,28], which have been shown to interact differently with soil components than glycosolated misfolded, aggregated prions [10]. Additionally, other components in a tissue homogenate, such as brain, create a competitive matrix in which prions have to compete with other components for surface binding, which is more representative of the natural system than

recombinant substrates [9]. Previous investigations of the soil-prion binding using whole brain homogenates containing mouse and hamster-adapted prions have attempted to quantify the amount of PrP^{RES} bound to soil [3,9,197]. But PrP^{RES} does not necessarily correlate with prion infectivity and studies estimating infectivity using prion-bound soil fractions have produced conflicting data. Soil-bound prions apparently increase infectivity upon oral inoculation [21], but decrease infectivity upon intracerebral (i.c.) inoculation [189]. Much remains to be understood about the specific interactions of soil and prions and how infectivity is increased by adsorption.

Conclusion

Despite the many advances in prion research, questions remain regarding conversion to the misfolded form, transmission dynamics, protein conformation, host environment, and species barriers. Yet, the protein-only hypothesis has held up against scrutiny, shifting the disease agent paradigm to include proteins as infectious agents.

As CWD continues to expand across North America, questions about prion persistence and transmissibility in the environment persist. Evidence suggests that soil acts as a prion reservoir allowing for efficient indirect contamination. Persistence, binding capacity and infectivity are expected to vary with environmental factors including soil type, environment, time and possibly prion strain stability. Additionally, behavioral ecology of the host will inform the risk of exposure, the rate and dose of prions ingested, and the rate of prion deposition in the environment. Studies investigating the transmission ecology and the other unique aspects of prions will help form a more complete picture of CWD as a disease.

Introduction to work in this Dissertation Research

The main objective of the current research is to gain a greater understanding and ability to measure the behavior and transmission dynamics of CWD prions in the environment. **The overall hypothesis for the thesis is that prions deposited in the environment by CWD-infected animals interact with soil components, remain infectious and play in important role in the transmission ecology of CWD.** Utilizing diagnostic assays and transgenic mouse bioassay we addressed the following research needs:

Question 1: When prions interact with soil, what amount of prion infectivity is adsorbed to soil?

Variability in apparent infectious titers of prions when bound to soil has complicated attempts to quantify the binding capacity of soil for prion infectivity. Here, we quantify the prion adsorption capacity of whole, sandy loam soil (SLS) typically found in CWD-endemic areas in Colorado; and purified montmorillonite clay (Mte), previously shown to bind prions, by BioAssay of Subtracted Infectivity in Complex Solutions (BASICS). We incubated prion-positive 10% brain homogenate from terminally sick mice infected with the Rocky Mountain Lab strain of mouse-adapted prions (RML) with 10% SLS or Mte. After 24 hours samples were centrifuged five minutes at 200 x g and soil-free supernatant was intracerebrally inoculated into prion susceptible indicator mice. We used the number of days post inoculation to clinical disease to calculate the infectious titer remaining in the supernatant, which we subtracted from the starting titer to determine the infectious prion binding capacity of SLS and Mte. BASICS indicated SLS bound and removed ≥ 95% of infectivity. Mte bound and removed lethal doses (99.98%) of prions from inocula, effectively preventing disease in the mice. Our data reveal significant prion-binding capacity of soil and the utility of BASICS to estimate prion loads and

investigate persistence and decomposition in the environment. Additionally, since Mte successfully rescued the mice from prion disease, Mte might be used for remediation and decontamination protocols.

Question 2: Can aptamers, (short oligonucleotides), be used to detect and measure natural levels of prions in excretory and soil samples?

In collaboration with InfoScitex, we developed and conducted preliminary trials of a novel detection assay utilizing aptamers for PrP^{RES} detection. Aptamers, single-stranded short oligonucleotides, are functionally akin to antibodies with the capacity to bind target molecules with high affinity and specificity. The concept was to use aptamers to selectively bind PrP^{RES} in a sample then apply direct or amplified detection. For direct detection we used electrophoresis and western blot. Amplified detection attempted to use quantitative, or real time, polymerase chain reaction (rtPCR) to amplify PrP^{RES} bound aptamers as a proxy for the presence or absence of prions. Our objective was to develop this assay to increase the detection limit of PrP^{RES} in environmentally relevant samples. We used PrP^{RES} positive and negative brain homogenates from elk and transgenic mice for assay development. Preliminary results from western blot experiments were mixed, with successful detection of some prion strains but not all. Experiments using rtPCR were susceptible to the inhibitory effect of environmental samples resulting in both false positive and negative results. Additionally, evidence of non-specific binding in elk brain homogenate suggested an affinity for an unknown molecule resulting in false positives. We believe that a negative-selection step in future aptamer development would resolve this issue. Further studies are required, but our results suggest that aptamers may be an option for future PrP^{RES} detection assays to address existing limitations in prion research.

Question 3: Can we improve the detection limit to accurately detect sub-clinically infected individuals on the landscape?

Currently, prion protein immunohistochemistry (IHC) of brain tissue is the gold standard for CWD detection. However, this method may be insensitive to early or sub-clinical cases of CWD that may play an important role in disease transmission ecology. Alternatively, the serial protein misfolding amplification assay (sPMCA) is a reliable detection assay and can be run in replicate for higher accuracy. This study compared the efficacy of IHC to sPMCA for the detection of low prion titers in naturally infected CWD-positive elk. By implementing a hierarchical Bayesian model, we estimated the specificity and sensitivity of all tests conditional on simultaneously estimated disease states. IHC test results were modeled as a Bernoulli trial while sPMCA test scores arose from a modified survival process across amplification cycles. Our results suggest that sPMCA of the obex is more sensitive (95%) in the detection of CWD prions than IHC of the obex (71%). Only through evaluation of multiple tissues does IHC sensitivity equal sPMCA. sPMCA can also return unbiased results in a shorter time frame than IHC, which requires specialized microscopy. Prevalence estimates of CWD in this free-ranging population were estimated at 21.62% as compared to a previously reported prevalence of 12.4%. Our data show a previously unidentified sub-clinical prion-positive elk population that could represent silent carriers and a source of prion shedding into the environment.

Question 4: What are the dose, exposure risk and transmission ecology of CWD prions in soil?

Experimental studies of indirect transmission of CWD from environmental PrP^{CWD} contamination are required to understand the disease ecology, epidemiology and overall maintenance of the disease in wild populations. The goal of this study was to 1) investigate the

role of indirect transmission by exposing PrP^{CWD} susceptible transgenic mice to PrP^{CWD} contaminated soil, 2) estimate average soil ingestion by laboratory mice, 3) estimate annual exposure to prions from chronic environmental exposure of prion contaminated soil and 4) evaluate the dose response of a single dose compared to chronic exposure. A prion-soil titration curve was created by orally inoculating mice with one of 5 dilutions of PrP^{CWD}-positive elk brain homogenate with 10% whole soil in sucrose. Second, additional groups of mice were either orally inoculated with, or housed on, soil originating from captive cervid research facilities where CWD occurred in herds. These soils, one from the Colorado Division of Parks and Wildlife research facility and the other from the Wyoming Fish and Game research facility, were considered "naturally contaminated" with PrP^{CWD}. Data from time point sacrifices and clinically ill mice indicate mouse bioassay successfully demonstrated the presence of prions in naturally contaminated soil samples. We used bioassay findings to also estimate the amount of infectious PrP^{CWD} consumed by mice housed on soil, and to demonstrate dose response of chronic exposure verse a single oral dose.

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CHAPTER 2:

ESTIMATING PRION ADSORPTION CAPACITY OF SOIL BY BIOASSAY OF SUBTRACTED INFECTIVITY FROM COMPLEX SOLUTIONS (BASICS)[1]

SUMMARY

Prions, the infectious agent of scrapie, chronic wasting disease and other transmissible spongiform encephalopathies, are misfolded proteins that are highly stable and resistant to degradation. Prions are known to associate with clay and other soil components, enhancing their persistence and surprisingly, transmissibility. Currently, few detection and quantification methods exist for prions in soil, hindering an understanding of prion persistence and infectivity in the environment. Variability in apparent infectious titers of prions when bound to soil has complicated attempts to quantify the binding capacity of soil for prion infectivity. Here, we quantify the prion adsorption capacity of whole, sandy loam soil (SLS) typically found in CWD-endemic areas in Colorado; and purified montmorillonite clay (Mte), previously shown to bind prions, by BioAssay of Subtracted Infectivity in Complex Solutions (BASICS). We incubated prion positive 10% brain homogenate from terminally sick mice infected with the Rocky Mountain Lab strain of mouse-adapted prions (RML) with 10% SLS or Mte. After 24 hours, samples were centrifuged five minutes at 200 x g and soil-free supernatant was intracerebrally inoculated into prion susceptible indicator mice. We used the number of days post inoculation to

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clinical disease to calculate the infectious titer remaining in the supernatant, which we subtracted from the starting titer to determine the infectious prion binding capacity of SLS and Mte. BASICS indicated SLS bound and removed ≥ 95% of infectivity. Mte bound and removed lethal doses (99.98%) of prions from inocula, effectively preventing disease in the mice. Our data reveal significant prion-binding capacity of soil and the utility of BASICS to estimate prion loads and investigate persistence and decomposition in the environment. Additionally, since Mte successfully rescued the mice from prion disease, Mte might be used for remediation and decontamination protocols.

INTRODUCTION

Prions are infectious agents of transmissible spongiform encephalopathies (TSEs) [1]. Misfolded, pathologic isoforms (PrP^{Sc}) of the normal mammalian prion protein (PrP^{C}) associate with prion infectivity, generally resist protease degradation, and often form insoluble, amyloidogenic aggregates [2]. Prions are capable of horizontal transmission between animals and indirect transmission from contaminated environments [3-10]. For reasons that remain unclear, indirect environmental transmission of prions appears to be limited to scrapie and chronic wasting disease (CWD) prions, and does not appear to be an ecological component of bovine spongiform encephalopathy (BSE) or other TSEs. This phenomenon may relate to scrapie and CWD sharing similar lymphotropic, shedding and transmission characteristics [11-13]. Infectious prions are likely deposited into the environment through alimentary shedding [14,15], placental material [16], antler velvet deposits [17] and the decomposition of prion-positive mortalities [5]. Once in the environment, studies have shown PrP^{Sc} to adsorb strongly to soil components [18-20], remain infectious [21-23] and persist for years [5,7,21,24]. Indirect transmission most likely occurs through incidental and geophagic ingestion of soil or other

contaminated fomites, as well as deer sign-post behavior such as scraping and marking overhanging branches [5,8,25].

Experimental evidence suggests that the particularly strong adsorption relationship of prions to soil colloids, or clays (defined as particles < 4 μm), may be responsible for the longevity in the environment [18,19]. Studies have shown percent-clay content of soil significantly influences the cation exchange capacity of soil and its overall negative charge [26]. Electrostatic and hydrophobic interactions between the prion protein and clay are thought to mediate this non-specific adsorption activity [27-30]. Specifically, montmorillonite (Mte), the most commonly occurring smectite clay, has been most implicated in the adsorption of prions in the environment [31]. Mte is a 2:1 phyllosilicate clay consisting of 2 tetrahedral silica composed molecules flanking one octahedral aluminum composed molecule, forming a sheet. An interlayer space exists between sheets capable of expanding to > 2 nm depending on the cationic concentration of the solution. It has been hypothesized that prions may enter this interlayer area like other proteins. However, Johnson et al. [31] did not find evidence of this in their experimental system and other studies suggest extensive protein unfolding would be required [32], which is unlikely for PrP^{Sc}. Mte is prevalent throughout the US mountain west, including CWD-endemic areas [33,34]. Models suggest that the prevalence of Mte at a landscape level may explain and predict CWD prevalence, which can exceed 20% in free-ranging cervids [33,35].

Other soil components such as organic material, quartz, tannins and humic acid have also been implicated in prion adsorption [19,29,31,36-39]. Whole soil also includes highly reactive humic substances, which have large specific surface areas and high binding capacities [40]. Humic acid can coat mineral surfaces imparting a net negative charge [41]. However, due to the

unknown tertiary structure of PrP^{Sc}, specific interactions and adsorption dynamics to soil and humic substrates have yet to be identified.

The robust adsorption relationship between the prion protein and soil has proven difficult to measure or reverse, limiting prion detection sensitivity, estimation of prion adsorption capacity of soil [19,29,31,42] and general progress in studying prions in the environment. Additionally, prion detection in soil has been successful only in laboratory experiments using a variety of different methods including antibody labeling [38], electrophoresis [37], bioassay [22], detergent extraction [36] and protein misfolding cyclic amplification (PMCA) [22,43].

To date, hypothesized soil interactions have largely been demonstrated with recombinant prion proteins [27,29,36], which probably interact differently with soil than glycosolated misfolded, aggregated prions. Previous investigations of the soil-prion relationship using whole brain homogenates containing mouse and hamster adapted prions have attempted to quantify the amount of PrP^{Sc} bound to soil [19,31,44]. But PrP^{Sc} does not necessarily correlate with prion infectivity and studies estimating infectivity using prion-bound soil fractions have produced conflicting data. Soil-bound prions apparently increase infectivity upon oral inoculation [22], but decrease infectivity upon intracerebral (i.c.) inoculation [23]. To circumvent these issues, and more accurately quantify infectious prion binding capacity of soil, we developed a converse assay. We investigated the adsorption capacity of prions to soil using an infectivity subtraction assay of titrated prion strains. This methodology allows measurement of unbound and unadulterated prions instead of prions bound to soil, which can alter infectivity [22,23]. We calculated the adsorption capacity of two soil types, a whole Colorado sandy loam soil (SLS) and pure montmorillonite clay (Mte) by assaying residual infectivity of supernatants from prion-soil matrices using TgA20 mouse bioassay [45]. SLS bound over 95% of prion infectivity and Mte

bound over 99.99% prion infectivity. These data promote BASICS as an effective tool for quantifying prion adsorption to soil as a function of infectivity and Mte as a potential compound for bioremediation of prion-contaminated solutions. We further propose that BASICS can improve estimates of landscape contamination that might exist in scrapie or CWD-endemic areas, thereby enhancing our understanding of the larger issues of environmental prion persistence.

METHODS

Mice

TgA20 mice over-expressing mouse PrP^c were generated as previously described [45] and allowed for quantitative LD_{50} infectivity analysis [46-48], defined as the prion dose that kills half of inoculated mice.

Ethics statement

Mice were bred and maintained at Lab Animal Resources, accredited by the Association for Assessment and Accreditation of Lab Animal Care International, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University (Protocol ID: 09-1580A). Intracerebral inoculations were performed under Isoflurane anesthesia, and mice euthanized using CO_2 inhalation to effect followed by decapitation. All efforts were made to minimize suffering.

Soil

Whole SLS used for this study was sourced from a private ranch in Southern Colorado located on the eastern side of the Rocky Mountains and within game management unit 861 which continues to test negative for CWD in free-ranging cervid populations [49]. Soil was collected with the land owner's (A. C. Wyckoff) permission, no additional permissions or

permits were required for the described field studies. SLS was passively air-dried, serially sifted first through a 1 cm sieve to remove rocks and debris, then through a 2 mm sieve and autoclaved (dry soil, 90 min at 120°C) to reduce incidental biotic agents naturally present in soil. Montmorillonite (powdered Western Bentonite) was sourced from Panther Creek, Co and supplied by Ward's Natural Science (San Luis Obispo, Ca).

Soil Analyses

Soil classification analysis of whole soil was conducted by the Colorado State University Soil, Water and Plant Testing Laboratory (Fort Collins, Co). X-ray diffraction mineralogy analysis of whole soil was conducted by K-T GeoServices, Inc. (Gunnison, Co). Whole soil analysis included XRD weight percentage for bulk (whole rock) and clay fraction (< 4 μm), pH, percent organic material, and soil texture classification of basic elements (Table 2.1).

Table 2.1. Soil Component Analysis

Mineral	***Whole Soil***[a]	***Mte***[a]
Quartz	35.9	
K-Feldspar	9.3	
Plagioclase	38.3	
Amphibole	1.3	
Calcite	1.3	
Pyrite	1.6	
Hematite	0.8	
R0 M-L I/S 90S[b]	2.2 (19.1)[c]	100.0
Illite & Mica[b]	7.7 (67.0)	
Kaolinite[b]	1.4 (12.2)	
Chlorite[b]	0.2 (1.7)	
Total	100.0 (11.5)[d]	100.0
Soil Characteristics		
Texture class	Sandy Loam	clay
% Sand	72.0	NA[f]
% Silt	14.0	NA
% Clay	14.0	100.0
Ph	7.5	9.9
EC (mmhos/cm)[e]	4.6	NA
% Organic Material	3.6	0

[a] % weight of whole SLS
[b] clay classification
[c] % of total clay weight
[d] clay weight % of total
[e] electrical conductivity (EC), measurement of salinity
[f] NA, not applicable

The following definitions were used for clay mineral classification: *Mixed-Layer Illite/Smectite* – A clay mineral group containing interlayered or interstratified Illite and Smectite. Mixed layer type was identified by the minerals involved (Illite and Smectite), the type of order or stacking along the Z-axis (random or not ordered), and the proportions of the minerals involved (10% Illite and 90% Smectite). *Illite and Mica* – Common non-expanding

minerals which are hydrated silicates containing potassium, silica and aluminum. *Kaolinite* and *Chlorite* – Common non-expanding hydrous aluminum silicate clay minerals. Montmorillonite clay was not further analyzed, specifics were obtained from the Material Safety Data Sheet (MSDS) sheet provided by the supplier.

Sources and Preparation of Prion Inocula

The Rocky Mountain Lab passage 5 strain of mouse-adapted scrapie (RML5) was previously described [50]. We derived the TgA20RML strain by passaging RML5 into TgA20 mice, resulting in inoculum with approximately one log lower infectivity titer compared to the original RML5 (see Table 2.2).

Table 2.2. Incidence and infectivity titers of prion inocula before and after adsorption

Inoculum	Adsorbed to	Incidence[a] (mean ± SD DPI[b])	Infectivity Titer[c]			% Bound
			Input[d]	Unbound	Bound	
NBH	SLS	0/2 (non-clinical at 250)	0	0	0	0
TgA20RML	nothing	4/4 (82 ± 3.4)	1.70	NA[e]	NA	NA
	SLS	6/6 (99 ± 6.4)	1.70	0.06	1.64	96.45
	Mte	0/7 (non-clinical at 200)	1.70	0	1.70	100
RML5	nothing	4/4 (73 ± 13.5)	14.8	NA	NA	NA
	SLS	7/7 (87 ± 6.6)	14.8	0.68	14.1	95.32
	Mte	1/5 (109, 4 mice non-clinical at 200)	14.8	<0.0032[e]	≥14.8	99.98

[a] number of terminally ill mice/number infected
[b] DPI, days post infection
[c] x 10^4 mean LD_{50} after 24h @ 23°C. All SDs ≤ 0.001 x 10^4
[d] Initial titer of inocula prior to adsorption
NA, not applicable
[e] below linear range of bioassay

Brain homogenates of clinically ill mice were prepared to 10% dilution in PMCA buffer (4 mM EDTA, 150 nM NaCl in PBS) and further diluted to 1% into similarly prepared 10% TgA20 normal brain homogenate (NBH) as previously described [51].

BioAssay of Subtracted Infectivity from Complex Solutions (BASICS)

We performed an infectivity subtraction assay to estimate binding capacity of SLS and Mte soil (Figure 2.1). We prepared 10% w/vol soil solutions by adding dry soil to previously prepared 10% brain homogenates (e.g. 30 mg dry soil added to 270 μl homogenate). All inocula,

with and without soil, were incubated at 23°C for 24 hours on a rocker to balance maximal binding in a competitive matrix with the decomposition of brain homogenate [19,28,31]. Samples were centrifuged for 5 min at 200 x g (Accuspin Micro, Fisher Scientific, Waltham, Ma) to clarify solutions of soil particles, thereby subtracting prion infectivity bound to soil or Mte from prion infectivity remaining in supernatant. Inoculation groups included non-soil treated TgA20RML and RML5 to establish baseline infectivity titers, experimental treatments of TgA20RML and RML5 with SLS or Mte soil, and a negative control of NBH with SLS.

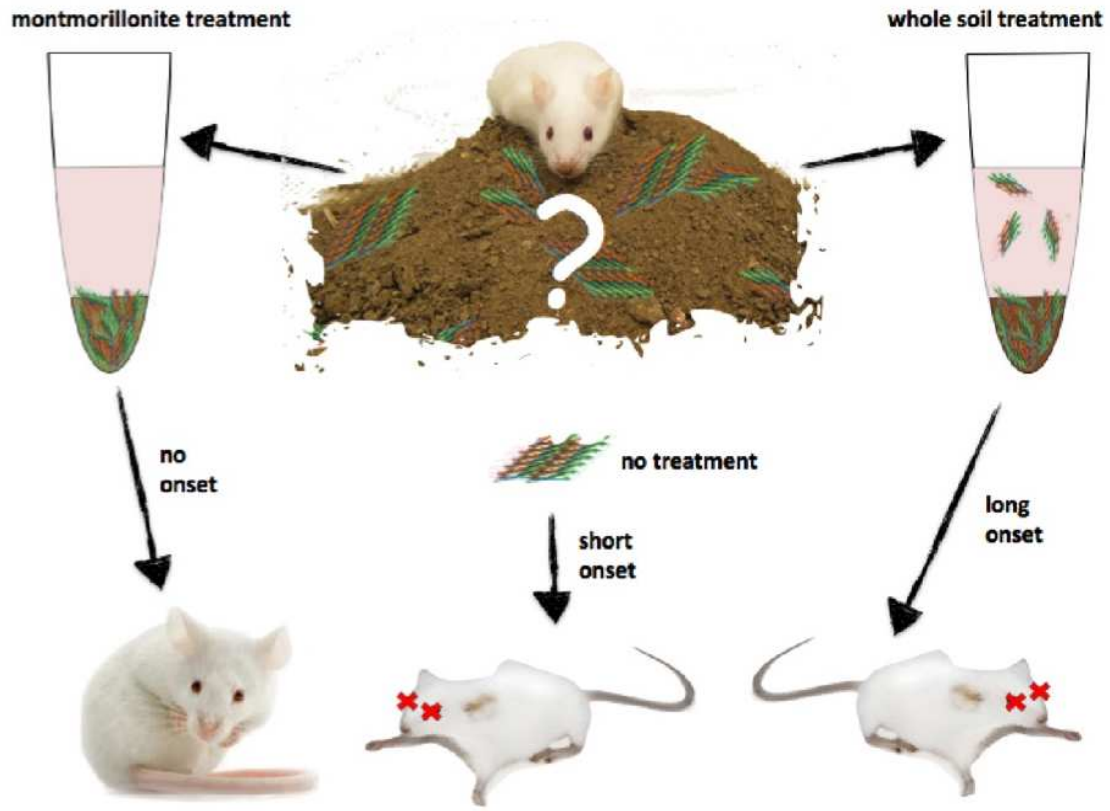

Figure 2.1. Visual Schematic of BASICS. To determine prion binding capacities of Mte and SLS, we incubated known titers of RML prions with or without Mte or SLS for 24 h at 23º C. A brief, low-speed centrifugation separated bound prions in the pellet from unbound prions in the supernatant. Degree of prion binding is then measured by bioassay in susceptible mice, subtracting supernatant titers from Mte (virtually no disease onset) or SLS (long onset) bound samples from control RML (short onset) supernatant titers.

Anesthetized mice were intracerebrally inoculated with 30 µl of inoculum (with 1% Pen-Strep added) as previously described [51]. Each treatment groups consisted of 5-7 mice. Onset of clinical disease was measured by scoring mice from normal (0) to exhibiting terminal clinical signs (4) for 7 different clinical signs including ataxia, akinesia, hyperactivity (0-3 scale), extensor reflex, tail rigidity (0-2 scale), weight loss and tremors. Mice receiving a composite score of 9 or greater, a single clinical score of 4, or exhibiting paralysis were euthanized and days post inoculation (DPI) to clinical disease recorded. DPI was used to calculate log infectivity titers of each inocula based on previous LD_{50} determinations for RML in TgA20 mice [2,45,52]. We used the linear equation y=11.45-0.088x (y, logLD_{50} per gram of brain; x, incubation time in DPI to terminal disease) to calculate infectivity titers as outlined in Reed and Muench [48]. Several non-clinical mice from each Mte-inoculated group were also euthanized after 130 DPI, and 200 DPI (the end of the study) to test their brain tissue for sub-clinical levels of prions by serial protein misfolding cyclic amplification (sPMCA). Brains tissues were collected from all mice for western blot (stored at -80°C) and a subset of mouse brains were also sampled for histological analysis (2/3 of brain was fixed in 10% formaldehyde, remaining section was frozen). Statistical analysis of Kaplan-Meyer survival curves and Student's t-tests of log infectivity were conducted using Prism 5 (GraphPad, La Jolla, Ca).

sPMCA and Western Blotting

Brain tissues of clinical and non-clinical mice, as well as samples of each inoculum were tested by western blotting before and after sPMCA. Prior to assay, brain tissues collected from mice were homogenized following the methods of Meyerett et al. [51]. sPMCA amplification substrate consisted of 25 µl of 10% TgA20 NBH combined with 25 µl of sample in 0.2 ml tubes. Tubes were sealed with parafilm, loaded into a holding tray and placed in a 37°C water bath in

the Misonix 4000 sonicator horn (Qsonica Inc., Farmingdale, NY). Samples were sonicated at approximately 200 watts (70% max power) for 40 sec every 30 min for 24 h, constituting one round. For each subsequent round, 25 µl of each sample from the previous round was added to 25 µl of fresh NBH. Duplicates of each sample were subjected to 6 rounds of PMCA to balance desired sensitivity (>80% of 10^{-7} dilution prion samples detected positive) and specificity (>98% of NBH samples remain negative) of the detection assay. Each group of samples was processed with at least five NBH negative controls and one positive plate control (CWD-positive elk brain homogenate E2, 1:10,000).

For visualization by western blot, 18 µl of sample was digested with 2 µl of 50 µg/ml proteinase K (PK, Roche, Basel, Switzerland) for 30 min at 45°C. The reaction was stopped by adding lithium dodecyl sulfate sample loading buffer (Invitrogen, Carlsbad, Ca) and boiling samples for 5 min at 95°C. Samples were electrophoresed through 12% sodium dodecyl sulfate polyacrylamide gels (Invitrogen) then electro-transferred to Immobilon P^{SQ} PVDF membranes (Millipore, Billerica, Ma) in transfer buffer (Invitrogen). Membranes were blocked for 1 hr with 5% nonfat milk in PBS with 0.1% Tween 20, and incubated overnight at 4°C in Superblock (Pierce, Waltham, Ma) with HRP-conjugated anti-PrP Bar-224 monoclonal antibody (SPI bio) diluted 1:20,000. Blots were washed 6 x 10 min in PBS with 0.2% Tween 20 before visualizing proteins using Immobilon chemiluminescent substrate (Millipore) and a Fujifilm LAS 3000 gel documentation system.

Immunohistochemistry

Dissected tissues were prepared and stained for PrP^{Sc} detection as previously described [51] with the following modifications. Briefly, tissues were treated with DAKO target retrieval solutions (DAKO, Carpinteria, Ca), then with formic acid to degrade PrP^{c}. PrP^{Sc} was labeled

with anti-PrP BAR224 followed by incubation with secondary EnVision HRP-conjugated anti-mouse antibody that was visualized with chromagen 3-Amino-9-ethylcarbazole (DAKO). Hemotoxylin and Glial fibrillary acidic protein (GFAP) stain of activated astrocytes was performed by the Colorado State University Histology and Diagnostic Laboratory as previously described [51]. Briefly, slides were treated with DAKO target retrieval solution then treated with primary anti-GFAP rabbit antibody at 1:100 (Cell Marque, Rocklin, Ca). Secondary anti-rabbit-goat biotinillated antibody was used with (BioGenex, San Romano, Ca) Enhanced Alkaline Phosphatase Red Detection Kit (Ventana, Tucson, Az).

RESULTS

In this study we collected soil from an area in southern Colorado with similar soil composition to CWD-endemic areas but with no reported cases of CWD. Soil component analysis revealed clay content similar to that found in areas of Colorado exhibiting high prevalence (Table 2.1). Specifically, the smectite clay Mte, previously shown to avidly bind prions [31] constituted approximately 2% of total soil and 19.1% of total clay content in our samples.

Incubation of 1% TgA20RML and 1% RML5 prions with either SLS or Mte significantly reduced the bioassay infectivity, resulting in delayed clinical disease ($p < 0.05$, Figure 2.2 and Table 2.2). Specifically, SLS incubation reduced the bioassay infectivity of the TgA20RML by 28.2 fold, a 96.5% reduction in infectivity. Infectivity of the same inoculum incubated with Mte was below bioassay detection limits (130 DPI [46]), resulting in all mice surviving to the end of the study at 200 DPI with no clinical signs of disease. Likewise, the infectivity of the RML5 inoculum was reduced by 21.4 fold, or 95.3%, after incubation with SLS. The mean binding capacity of SLS for RML prions in both inocula was $8.13 \times 10^5 \pm 1.2$ LD_{50} units/g soil.

Incubation of RML5 with Mte resulted in a near total removal of infectivity with only one mouse becoming ill, equating to at least a 1380-fold reduction in infectivity. Mte completely removed lethal doses of TgA20RML prions (1.7 x 10^4 LD_{50} units), indicating that its RML5 binding capacity is at least 5.63 x 10^8 LD_{50} units/g of Mte.

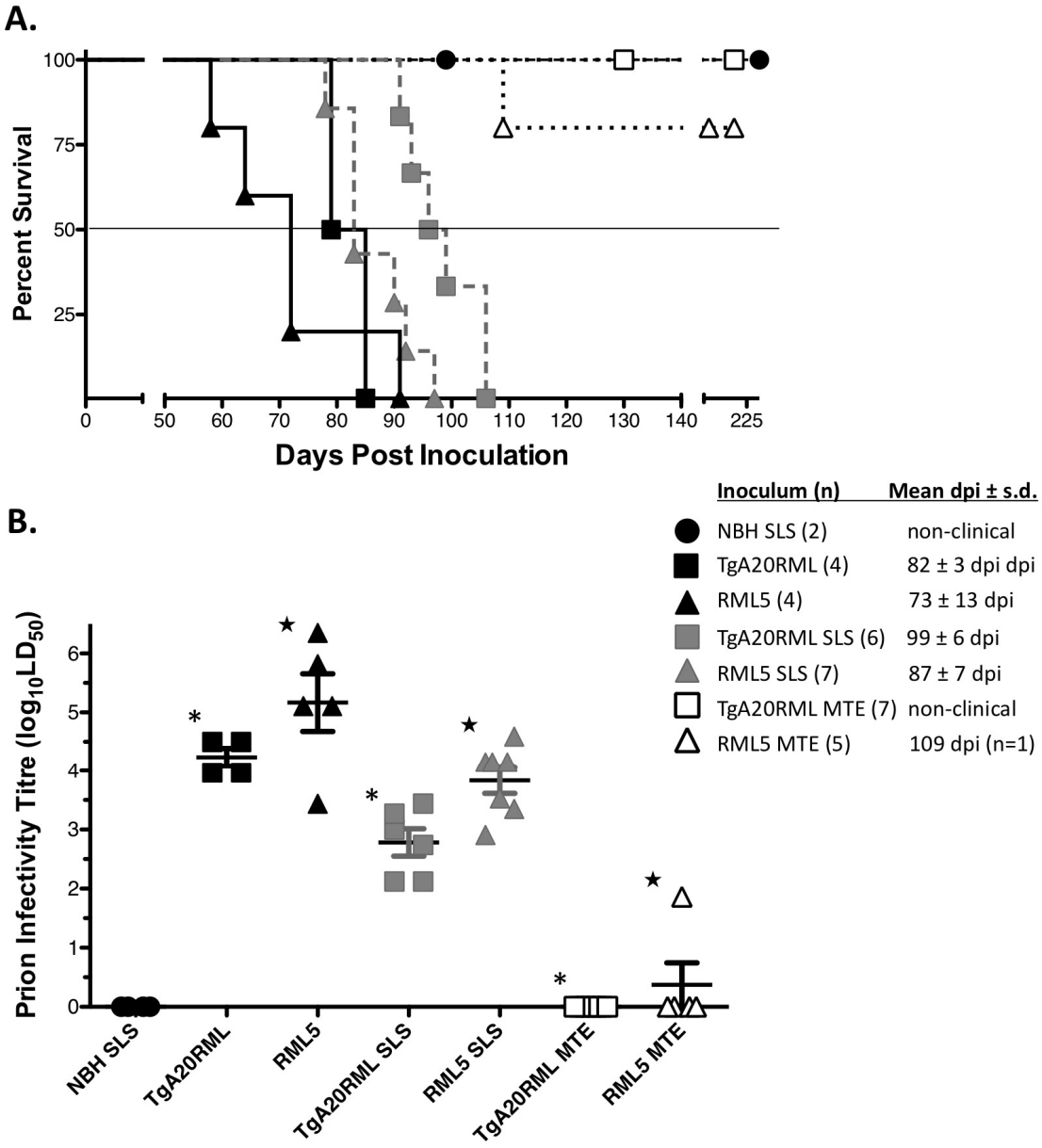

Figure 2.2. Survival of TgA20 indicator mice following i.c. inoculations. (**A)** Kaplan-Meyer survival curve of 7 treatment groups demonstrates the delayed disease onset in mice infected with SLS treated inocula (grey squares and triangles), and the nearly complete abrogation of disease in mice infected with Mte treated inocula (open squares and triangles)

compared to control mice infected with neat inocula (black squares and triangles). Mice infected with SLS-treated negative brain homogenate (black dots) did not exhibit any disease. **(B)** Disease onset of each group were clustered and consistent with reduced LD_{50} values. Inoculum type was significantly different ($P < 0.05$) than their respective treatment, (= significant difference between TgA20RML treatments, = significant differences between RML5 treatment groups). Data is presented with treatment group median and s.d. error bars.

To determine whether non-clinically sick mice replicated sub-clinical levels of prions, we attempted to amplify minute quantities of prions from their brains using sPMCA. We detected prions in 2/7 brains from non-clinical mice inoculated with TgA20RML pre-adsorbed with Mte and 2/4 brains from non-clinical mice inoculated with Mte-adsorbed RML (Figure 2.3, Table 2.3).

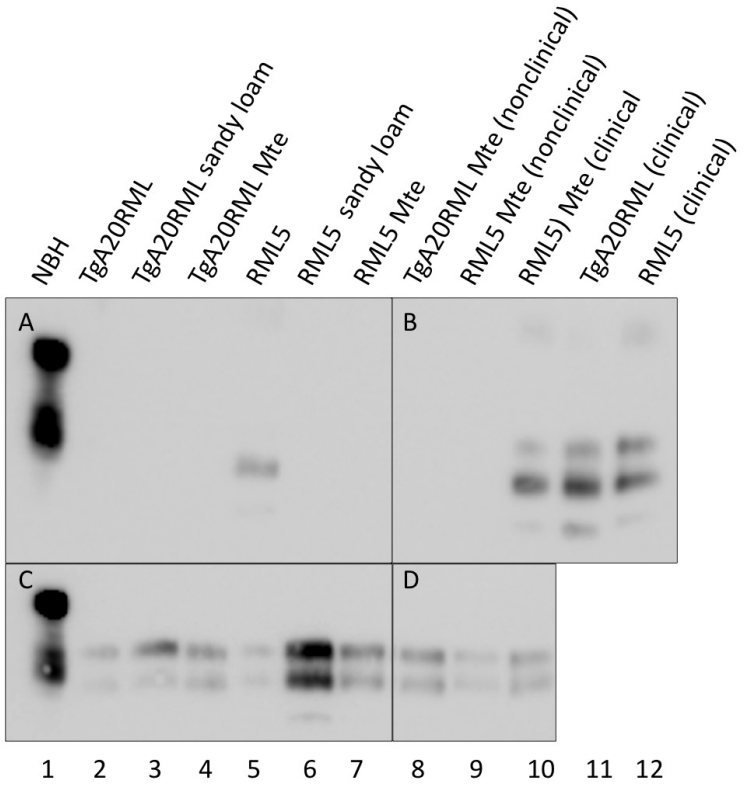

Figure 2.3. Representative PK digestion and Western blot analyses of inocula and inoculated animals. All samples were PK digested except lane 1 **(A)** PrP^{Sc} content of inocula were below western blot detection levels with the exception of RML5 (lane 5). **(B)** Brain homogenates from non-clinical experimental animals (lanes 8-9) were also negative by western blot, however, samples from clinically ill mice showed PrP^{Sc} (lanes 10-12). **(C)**With 6 rounds

of PMCA, PrP^{Sc} was detected in all inocula, and **(D)** in non-clinical mouse brain tissues samples.

Table 2.3. Disease status and detection of prions in non-clinical mice inoculated with Mte-treated-inocula

Treatment Mice	**DPI**	**sPMCA +/-**
TgA20RML-Mte		
1	131	+
2	131	-
3	131	-
4	200	+
5	200	-
6	200	-
7	200	-
RML5-Mte		
1 (clinical)	109	+
2	131	-
3	131	-
4	200	+
5	200	+

These results suggested that sub-clinical levels of prions existed in some individuals despite the lack of clinical disease. To confirm this observation, we also investigated neuropathology in these mice and compared them to clinically ill mice. Histological examination for PrP^{Sc} deposits, spongiosis and astrogliosis revealed differences in histopathology between SLS-adsorbed prion-inoculated mice and the single Mte-adsorbed RML5-inoculated mouse that became clinically ill (Figure 2.4).

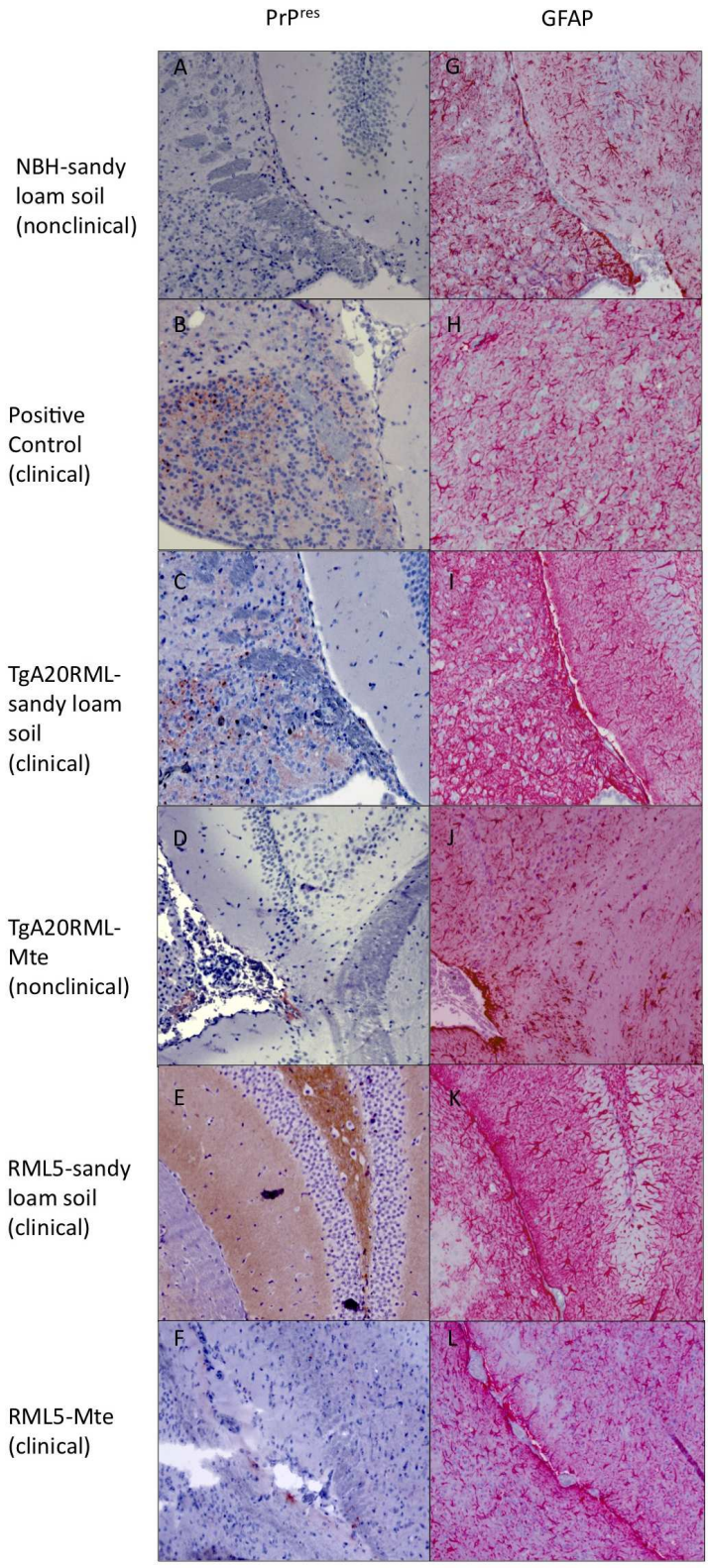

Figure 2.4. Representative Histology of TgA20 indicator mice. Selected examples of histological analysis using immunohistochemistry with PrP specific BAR224 Ab (reddish-

brown staining, panels A-F) and anti-GFAP antibody staining activated astrocytes (bright red, panels G-L) in hippocampal sections. (**A and G)** Negative control sections from mice inoculated with SLS-treated NBH exhibited no PrP^{Sc} staining or spongiosis and limited astrocyte activation. (**B and H)** Positive control sections from mice inoculated with RML5 revealed diffuse PrP^{Sc} staining and significant spongiosis and astrogliosis. (**C and I)** Sections from mice inoculated with SLS-treated TgA20RML resulted in limited PrP^{Sc} deposits, spongiosis and astrogliosis, while (**D and J)** sections from mice inoculated with Mte-treated TgA20RML revealed little or no scrapie neuropathology. (**E and K)** sections from mice inoculated with SLS-treated RML5 resulted in neuropathology similar to sections from TgA20RML treated mice (Band H). (**F and L)** Hippocampal sections from the only mouse to become ill with Mte treated RML5 showed limited PrP^{Sc} spongiosis and astrogliosis.

We detected no PrP^{Sc} or spongiform lesions and only mild astrogliosis in brains from non-clinical mice inoculated with control NBH (panels A and G) and Mte-adsorbed TgA20RML (D and J). We detected small deposits of PrP^{Sc} and spongiosis and slightly more astrogliosis in the brain of the lone clinically sick mouse inoculated with Mte-adsorbed RML5 (F and L). In contrast, we observed both diffuse and punctate PrP^{Sc} aggregates and mild to severe spongiosis and astrogliosis in brains from clinically ill mice inoculated with non-adsorbed prions (B and H) and whole soil-adsorbed TgA20RML (C and I) and RML (E and K). Together with the biochemical analysis, these data confirm prion infection in clinically ill mice, as well as sub-lethal infection in non-clinical mice, which we now call sub-clinically ill mice.

DISCUSSION

Environmental persistence and increased transmissibility of soil-bound prions remain poorly understood but extremely important aspects of both scrapie and CWD ecology. The use of SLS and Mte allowed us to model the complexity of prion binding in the natural environment, while estimating the relative contribution of a soil component previously shown to avidly bind prions. The use of RML in these studies allowed for LD_{50} calculations and quantitative statements of prion binding capacity of soil and Mte as a function of infectivity. Use of whole brain homogenate, as opposed to recombinant protein or enriched prions, accounts for the

complexity of the tissue and competitive matrix binding activity [19]. PrP^{Sc} is conceivably a small component of the brain matrix, and will compete with other proteins for binding sites on soil particles. Previous studies revealed the potential for increased adsorption if allowed to incubate for more than 24 hours [19], so we consider the adsorption measurements in our study to be an conservative estimate of the adsorption capacity of soil in a natural system. However, as previously mentioned, we sought to balance decomposition and microbial contamination of tissues with binding activity. Surprisingly, we observed a nearly one log reduction in infectivity of the positive control, non-adsorbed, inocula simply from an 24-hour incubation at room temperature. We also acknowledge that the behavior of RML in soil may not fully represent the behavior of scrapie or CWD in soil [44,53]. We used RML as a model system because RML titers have been previously determined. Other recent prion-soil binding studies used titered hamster scrapie strains to estimate [31] and quantify [23] prion binding capacity of soil. However, both studies involved inoculating soil bound prions, which exhibited different infectivity than equivalent doses of unbound prions and varied by inoculation route [23]. These factors potentially skewed estimates of infectious doses adsorbed to soil.

BASICS circumvents these problems by quantifying prion binding to soil by subtracting residual prion titers present after soil adsorption from initial prion titers before soil adsorption. We are currently titrating several other CWD field isolates and laboratory strains and will use BASICS to quantify binding capacity of relevant soil types to relevant prion strains.

We found a dramatic decrease in infectivity with a simple 24-hour soil incubation. The Mte treatment of TgA20RML bound sufficient amounts of infectious prions to prevent disease onset entirely. Similar binding was seen in RML5 samples, resulting in SLS binding 95.3% of infectious prions and the Mte binding $\geq$ 99.98%. These results suggest that the adsorption

capacity of the Mte, in these experimental conditions, lies somewhere between the TgA20RML and RML5 titers. If the Mte comprises the majority of smectite clay in the soil (90% of the 2.2% smectite, Table 2.1), then we calculate the maximal binding capacity of Mte present in the soil to be 98.51 x 10^4 LD_{50} units of RML per gram of soil. This amount comprises between 2% and 18% of the total prion binding capacity of soil that we observed for RML5 and TgA20RML, respectively. The estimated 2% binding capacity of Mte for RML5 correlates to the 2% Mte found in SLS, suggesting that the Mte is saturated with prion infectivity. The observation that the 2% of Mte correlates to binding nine-fold more TgA20RML infectivity (18% of total bound infectivity) in SLS also supports this interpretation, because TgA20RML titers were approximately nine times lower than RML5 titers. This would leave the remaining prions available for binding to other soil components such as other clays, quartz, humic acid or other organic material. Indeed, other soil components have been implicated in protein adsorption, including organic material, tannins, quartz [19,29,31,36-39], and competitive matrices have been shown to retard prion binding to soil [19]. Thus, we cannot completely disqualify the effects of small amounts of residual soil components remaining in solution after low-speed centrifugation that may bind prions and decrease their infectivity upon i.c. inoculation. But these effects are likely minor since centrifugation removed clay components, which we show here as others have previously, to be the major prion binders. If such effects exist, we again acknowledge that BASICS would conservatively estimate prion binding capacity of soil.

These data suggest that Mte is not the only factor determining prion binding capacity of soil. However, we propose that prions bind Mte with relatively high avidity and affinity compared to other soil components, whose prion interaction may be more reversible, creating equilibrium between prions bound to soil and free in solution. We hypothesize that Mte

concentrations in the soil dictate this equilibrium and likely result in residual infectivity in supernatants in our and other experimental systems [54,55] and possibly increased mobility in a natural system [56]. Indeed, the neuropathology and sPMCA data revealed sub-clinical levels of prion in the brain tissue of mice inoculated with Mte-adsorbed inoculum. Although the residual prions were not biologically relevant since the mice exhibited no clinical or pathological signs of scrapie, similar subclinical infections in wild cervid populations may contribute to ecologically relevant contamination, persistence and transmission.

Perhaps the most utilitarian finding of this study was the prevention of disease by pre-adsorbing prions with Mte. These data strongly promote Mte for prion remediation applications. Environmental prion mitigation looms as a potential desideratum for agriculture and wildlife management. However, options for degradation and removal of prions have shown limited efficacy [57-59]. Our results suggest that the binding of prions to Mte may be utilized for removing prions from liquids. Landscape application might not be feasible, but other decontamination or remediation applications may be possible in the medical, municipal and research sectors. For example, decontamination of blood, urine and components thereof, as well raw water in endemic areas and liquid waste in prion research facilities may be feasible.

In summary, we propose that although constituting a relatively small fraction of total soil, the high binding avidity and affinity of Mte results in high prion occupancy at or near saturation that may drive the likelihood of environmental prion contamination, persistence and transmission in nature, as has been previously suggested [33]. We are currently testing this hypothesis by experimentally increasing Mte concentrations in whole soil and using BASICS to assess the correlation to increased prion binding capacity of soil.

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CHAPTER 3:

DEVELOPMENT OF AN APTAMER DETECTION ASSAY FOR MULTIPLE STRAINS OF PRIONS

SUMMARY

Detection of infectious prions (PrP^{RES}) in environmentally relevant samples such as feces, urine and soil remains unreliable. Evidence suggests these samples may contain inhibitory factors that prevent existing assays from successful detection of PrP^{RES}, requiring a new technique for successful prion detection. In collaboration with InfoScitex, we developed and conducted preliminary trials of a novel detection assay utilizing aptamers for PrP^{RES} detection. Aptamers, single-stranded short oligonucleotides, are functionally akin to antibodies with the capacity to bind target molecules with high affinity and specificity. The concept was to use aptamers to selectively bind PrP^{RES} in a sample then apply direct or amplified detection. For direct detection we used electrophoresis and western blot. Amplified detection attempted to use quantitative, or real time, polymerase chain reaction (rtPCR) to amplify PrP^{RES} bound aptamers as a proxy for the presence or absence of prions. Our objective was to develop this assay to increase the detection limit of PrP^{RES} in environmentally relevant samples. We used PrP^{RES}-positive and -negative brain homogenates from elk and transgenic mice for assay development. Preliminary results from western blot experiments were mixed, with successful detection of some prion strains but not all. Experiments using rtPCR were susceptible to the inhibitory effect of environmental samples resulting in both false positive and negative results. Additionally, evidence of non-specific binding in elk brain homogenate suggested an affinity for an unknown molecule resulting in false positives. We believe that a negative-selection step in future aptamer

development would resolve this issue. Further studies are required, but our results suggest that aptamers may be an option for future PrP^{RES} detection assays to address existing limitations in prion research.

INTRODUCTION

The unusual nature of prions has challenged the concept of transmissible pathogens and created a formidable challenge for detection and study of the agent [1-3]. Currently, there are limited methods of detection for prions. These include immunodetection [4-6], bioassay in susceptible strains of laboratory mice [7,8], protein misfolding amplification assay (PMCA) [2] and the more recent real-time quaking-induced conversion (RT-QuIC) [9]. Immunoassay techniques are capable of rapid results but are considered to have a low sensitivity [2]. Bioassays allow for passage of transmissible material, modeling the disease and allowing for *in vivo* studies, but require use and housing of large numbers of animals, take months to complete and have low sensitivity. PMCA is the most sensitive of the detection assays, but the process of amplifying the positive signal in a sample requires amplifying the infectious agent itself, increasing biosafety concerns. RT-QuIC appears to be nearly as sensitive as PMCA and uses Thioflavin to measure amplification of non-infectious PrP aggregates [9]. Despite the respective drawbacks, each of these methods are extensively used, and have contributed to the understanding of prion diseases in many sample types. Certain types of samples remain extremely challenging or currently impossible assay, including blood, saliva, soil and other environmental samples. Additionally, with the exception of RT-QuIC, the *in vitro* detection methods also require antibodies to label the target molecule and antibodies currently available do not reliably distinguish between PrP^{C} and PrP^{RES} without PK digestion. This step risks the

digestion of PK sensitive, but disease relevant, forms of PrP^{RES} reducing the sensitivity of disease detection and limiting the diagnostic sensitivity.

Aptamers, single-stranded short oligonucleotides, are functionally akin to antibodies with the capacity to bind target molecules with high affinity and specificity [10], potentially providing a new technique to address existing limitations in prion research. Over the last 13 years, studies have shown aptamers to form complexes that selectively bind target molecules ranging from cell surface receptors to cytokines [11]. Similar to antibodies, these complexes demonstrate dissociation constants in the nano-molar range allowing for precise detection of target molecules [12,13]. The application of aptamers to the field of prion research is not entirely new, however, to date an aptamer-based prion detection assay has not been developed. Early work was conducted using recombinant hamster and other endogenous forms of PrP^{C}, however, these experiments failed to detect PrP^{RES} [14]. Much of the more recent work has shed light on conformational differences and binding sites for different forms of PrP^{C} and β-sheet rich conformations [12,15,16] but were unsuccessful in developing a PrP^{RES} detection test. The most successful prion related aptamer research was reported by Wang et. al., [17] however, the aptamer specificity was only reproducible for two human Creutzfeldt–Jakob disease (CJD) strains, several scrapie strains and a white tailed deer chronic wasting disease (CWD) strain, and results were not quantitative.

The objective of this study was to develop a bench-top detection assay utilizing PrP^{RES} specific aptamers and western blot or PCR to not only detect prions in a variety of sample types, including environmental samples, but to also successfully distinguish between strains through epitope or conformational differences. While we identified several candidate aptamers, we also observed inconsistent results with several different experimental protocols. We discuss the

potential of aptamers for ultrasensitive and strain specific prion detection and alternate methods of aptamer selection for future detection of prions.

METHODS

Biosecurity

Experiments using mouse-adapted scrapie (Rocky Mountain Laboratory strain; RML) [18], CWD field isolates and mouse passaged CWD were conducted in a biosecurity level 2 laboratory (BSL2) at Colorado State University. Experiments using bovine spongiform encephalopathy (BSE) or CJD material were conducted in a BSL3 laboratory under the supervision and permits of the Pierluigi Gambetti lab at the National Prion Disease Pathology Surveillance Center, Case Western Reserve University, Cleveland, Ohio.

CWD prions

CWD field isolates were derived from brain tissue from clinically ill Colorado captive elk. One hemisphere of each CWD-positive elk brain was homogenized to a 10% dilution with PMCA buffer (4 mM EDTA, 150 nM NaCl in PBS) as previously described [19] and aliquots were stored at -80 °C until needed. We also used 10% elk fecal homogenates prepared as previously described [20].

Mouse adapted prions

Brain tissue, serum and urine from TgA20 [21] mice inoculated with the RML strain of mouse prions and Tg5037(cerPrP) mice [22] inoculated with E2 CWD isolate or E2 serially passaged twice through Tg5037 mice (E2p2). Brain tissues were homogenized as previously described [23].

BSE prions

BSE samples were provided for western blot testing only, courtesy of the Pierluigi Gambetti lab. Both BSE samples used were positive by western blot (Figure 3.1).

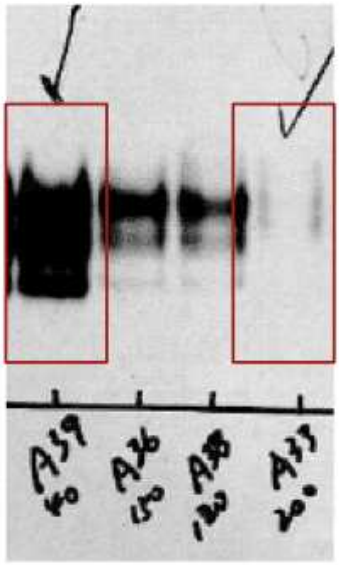


Figure 3.1. Immunoreactive western blot showing positive reactivity of BSE material used in this study (courtesy of Lui Ting Qing, Gambetti Lab, Case Western)

Tg(HuPrP) BSE prions

In addition to bovine BSE we also tested brain samples from BSE-infected human-PrP expressing transgenic mice. Samples from these animals were considered similar to variant CJD (vCJD) samples. These samples were only used for aptamer western blot and were also provided courtesy of the Pierluigi Gambetti lab.

Human CJD prions

CJD-positive human brain homogenate and cerebrospinal fluid samples (CSF) were provided for aptamer western blot testing by the Pierluigi Gambetti lab.

Negative tissue samples

Normal brain homogenate (NBH) was used from several sources; 1) perfused TgA20 (TgA20NBH) mice overexpressing mouse PrP^{C}, 2) perfused Tg5037 mice overexpressing cervid PrP^{C} as previously described (Tg5037NBH) [23], 3) unperfused mouse NBH (upNBH), 4) Negative elk brain (eNBH) obtained from a CWD free wild population in Montana, 5) Negative Tg1102(HuPrP) mouse (Tg1102NBH), and 6) Neg. human brain homogenate (1875NBH); Other, non-brain tissues included mouse urine and serum from both negative TgA20 and Tg5037 mice and neg. human CSF. All BSE, CJD and human samples were courtesy of the Pierluigi Gambetti lab at Case Western.

Animal work was approved by Colorado State University IACUC Protocol ID: 09-1580A, Approval Date: January 14,2010.

Isolation of Aptamers

Using a library of random ssDNA oligomers (DNA randomers) custom manufactured for InfoScitex by Sigma-Genosys (The Woodlands, Tx) [13], the DNA randomers were incubated with PrP^{RES} and then electrophoresed through a denaturing polyacrylamide gel matrix. Gel segments containing aptamer-PrP^{RES} complexes were excised and prion-bound aptamers recovered with 1.8 M NaSCN. Candidate aptamers were further selected by a high throughput aptamer isolation technique, rapid isolation of DNA aptamers (RIDA), developed by IST. RIDA allows for the rapid identification of high affinity and selectivity aptamers for the target molecule. In brief, the target PrP^{RES} protein was linked to silica beads and placed in a column. 1 ml of the candidate aptamer pool was run through the column overnight at 23 °C, unbound DNA oligomers were washed from column with a pH 8.0 PBS wash. PrP^{RES} bound aptamers were released from the protein with a 0 to 1.8 M gradient of NaSCN applied over 2 hours. The 0.1 ml

fractions containing the aptamers were collected and stored at -20 °C. The resulting candidate aptamers were amplified by PCR and sequenced. The project was conducted in three phases, Phase 1 (2009) was proof of concept with the production of 3 candidate aptamers, P1O1-P1O3. Phase 1 also included a modification of the aptamers, to optimize tails and respective primers for improved PCR amplification and reduced risk of primer dimmers. Aptamers with the modified tails were denoted with a "b" at the end of the name, eg. P1O2b would mean Phase 1 Oligo 2 modified version b. Phase 2 was a re-selection of aptamers, P2O1-P2O5, to try and improve specificity and sensitivity by PCR, and Phase 3 was testing BSL2 & 3 samples directly by WB. Representative aptamer structures are presented in Figure 3.2. Primer sequences for original and modified aptamers are outlined in Table 3.1.

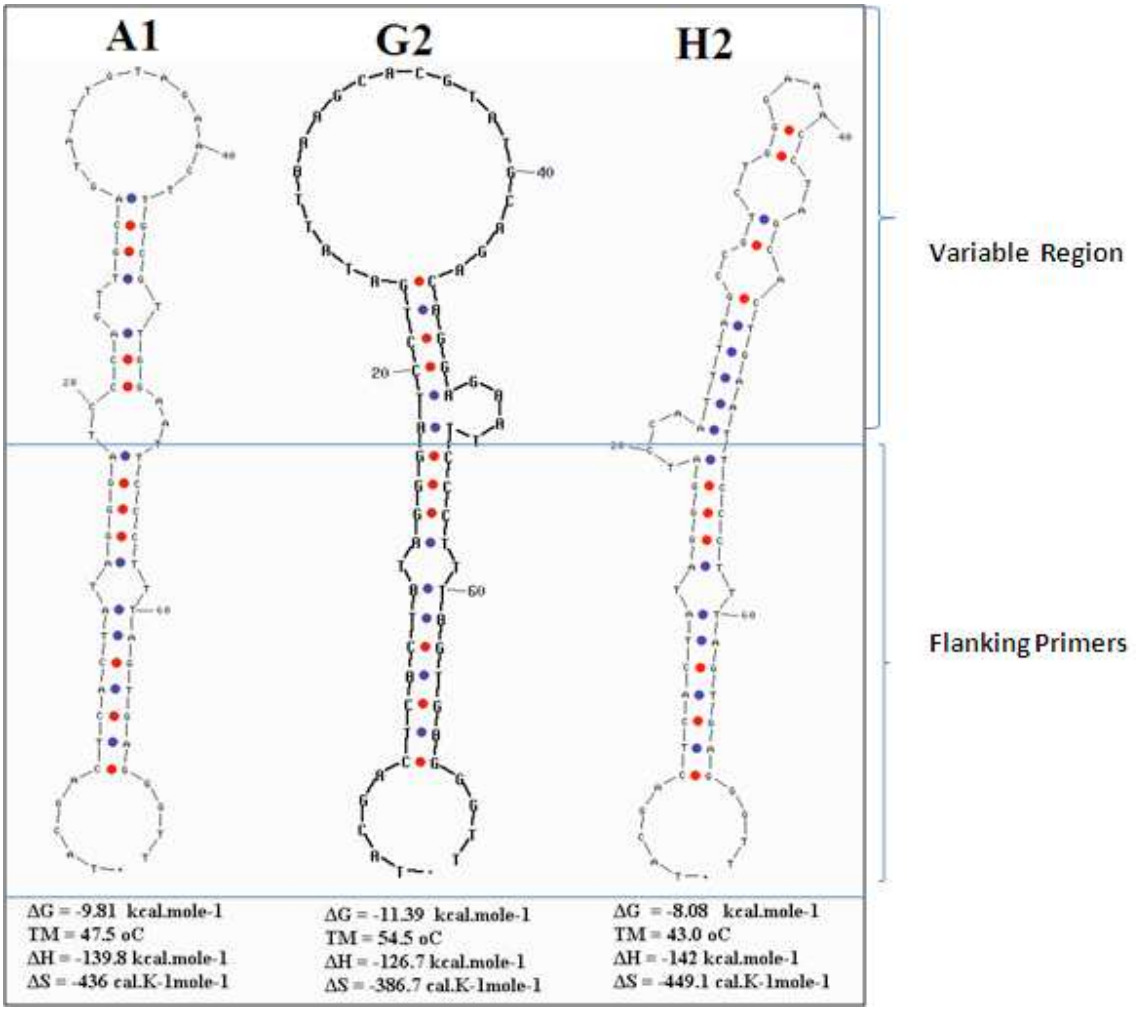


Figure 3.2. Representative aptamer structures and thermodynamic properties.

Table 3.1. Primer sequences.

Primer	Sequence
AP3	AACCCTCACTAAAGGGAATT
AP3b	AACCCTCACTAACCGGAATG
AP7	TACGACTCACTATAGGGATCC
AP7b	TACGACTCACTATCGGGATGC

Phase 1 & 2- Real time PCR

Samples were first treated with DNAse1 (Sigma-Aldrich, St. Louis, Mo.) at a 1:2 dilution for 10 minutes at 37°C to remove host DNA and prevent false positives. Batches of DNAse1 varied significantly and had to be titrated for each lot. The average concentration used was between 5 and 15 units/µl. After DNAse digestion samples were heated to 80°C for 10 minutes to deactivate DNAse. 8 µl of sample was incubated with 2 µl of 1 nM aptamer at 23°C for 20 minutes. Test samples included a positive rtPCR control of 1 pM untreated aptamer in master mix, negative mouse and elk brain homogenates for negative plate controls and duplicates of all samples. To remove unbound aptamers, samples were digested with 10 units DNAse for 2 minutes. PrP^{RES} bound aptamers were protected from digestion and remained in the sample. The DNAse was deactivated with 100 ng Proteinase K (PK) at 45°C for 10 minutes, and the PK was deactivated by boiling the sample at 95°C for 10 minutes. In a 96 well plate 2 µl of sample was mixed with 22.5 µl of master mix (MM). MM consisted of primers mixed with either SyberGreen Master Mix (BioRad, Hercules, CA) or SsoFast EvaGreen Master Mix (BioRad). Samples were run in a 96 well plate in an iCycler rtPCR machine (BioRad, iCycler) using the following cycle protocol: 3 minutes pre-melt at 95°C, 30 cycles of 20 seconds at 95°C, annealing and extension for 45 seconds at 50°C, melt temp cycle. See Figure 3.3 for a visual summary of the rtPCR approach. Samples were considered positive if they rose above the negative sample threshold.

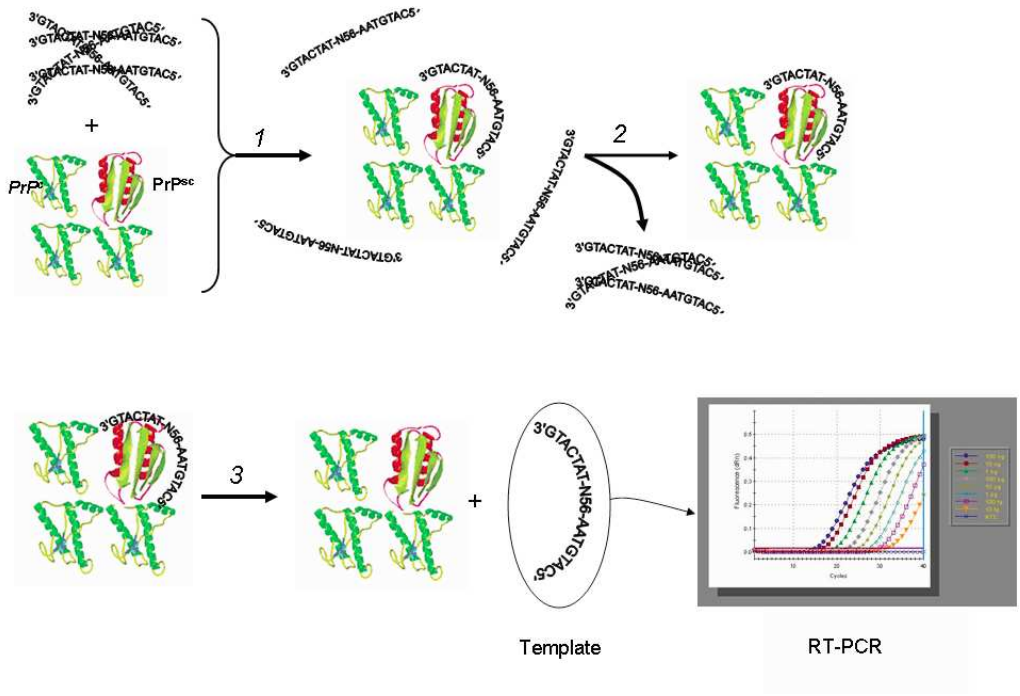


Figure 3.3. Schematic of detecting infectious prions using rtPCR of the DNA aptamers.

Due to inconsistent results with standard rtPCR we also tried combining the aptamer assay with superparamagnetic beads for enhanced amplification. We created an adapted protocol from [24]. Previous research suggests the beads bind prions allowing for enrichment by removing the bead-bound prions from solution with a magnet [24]. Briefly our adapted protocol was as follows: Combine 2 µl of either 1 nM aptamer with 8 µl of test sample, incubate at 23°C for 20 minutes. Prepare beads by washing in wash buffer (1 x PBS 0.5% Trition X-100), then resuspend in assay buffer (2 x TBS 1% Trition X-100 1% Tween). In a separate tube combine 5 µl of aptamer/test sample with 25 µl of beads and add another 150 µl of 1 x TBS. Place sample on a gentle mixer at 23°C over night. Wash beads three times with 1 x TBS, resuspend sample in assay buffer and place on shaker at 37°C for 20 minutes to melt aptamer off prion. Quickly remove sample from heat, place on magnet and remove supernatant containing aptamers.

Combine 2.5 µl of sample with 22.5 µl of master mix (MM) in a 96 well plate. Run rtPCR protocol as outlined above.

Phase 3 - Western Blotting

BSL3 experiments at Case Western Reserve University were conducted using the following protocol. Samples were PK digested at 37°C for 60 minutes (Thermomixer, Eppendorf). Human CSF was treated with 5 µl of 10 µg/ml PK in dH_20, all other samples were treated with 100 µg/ml. After digestion, samples were removed from heat and combined with 100 µl of 2X loading buffer, PK was deactivated by boiling for 5 minutes at 95°C. A subset of samples (mouse 1420, human 1399, BSE 33 and 39) were incubated with 15 ng of the biotinylated P2O1 aptamer at 23°C for 30 minutes to see affect of binding on size exclusion migration. Undigested positive samples were further diluted 1:20 in loading buffer. 15 µl of each sample was loaded in triplicate or quadruplicate into 18 well gels (see Table 3.2 for outline; BioRad Criterion 10-20% Tris-Glyicine 18 well) in 1X running buffer (Tris-Glycine SDS). 10 µl of a molecular weight ladder (Precision Plus protein standard BioRad) were loaded into the first lane of each gel. Electrophoresis was run at 80V for 20 minutes, then 100V for 120+ minutes. Gels were transferred (Transfer Blot Cell, BioRad) to membrane in 1X transfer buffer (Tris-Glycine) for 90 minutes at .36A. The membrane was incubated in blocking solution (5% nonfat dry milk in 1X TBS + 1% Tween) on rocker for 60 minutes at 23°C.

Blots were run in duplicate allowing for antibody (Ab) visualization as a positive control. Blot numbers 2 and 4 were incubated overnight at 4°C with a primary antibody combination of 6H4, 8H4, and 3F4 Ab (1:5,000 of each Ab in 1X TBS 1% Tween and 5% milk). We used a combination of the three antibodies to recognize three different prion strains. The blots were washed and incubated with a secondary HRP conjugated anti-mouse IgG antibody for one hour

at room temperature. The second set of blots, numbers 1 and 3, were incubated overnight at 4°C with 10 μg/ml biotinylated P1O1 aptamer. All blots were washed 4 times for 5 min with 1X TBS 2% Tween and 2x5 1X TBS with 0.1% Tween. Aptamer treated blots were incubated with Strepdaviden-HRP Conjugate (Sigma P3563 lot 108K8213) in 1X PBS with 0.05% Tween for 30 minutes. Blots were again washed 6 times for 5 minutes each in 1X PBS 0.1% Tween. Blots were treated with a chemiluminescent substrate and visualized by exposure to radiographic film.

Table 3.2. Western blot gel sample layout

Lane	**Gel 1 – P1O1 Aptamer**	**Gel 2 – Antibody**	**Gel 3 – P1O1 Aptamer**	**Gel 4 – Antibody**
1	TgHum1102NBH	TgHum1102NBH	TgHum1102NBH	TgHum1102NBH
2	TgHum1102NBH	TgHum1102NBH	*BSE 39	*BSE 39
3	TgHum1102NBH	TgHum1102NBH	ψBSE 39	BSE 39
4	*BSE tgHum1420	*BSE tgHum1420	BSE 39	BSE 39
5	ψBSE tgHum1420	BSE tgHum1420	BSE 39	BSE 39
6	BSE tgHum1420	BSE tgHum1420	- human CSF	- human CSF
7	BSE tgHum1420	BSE tgHum1420	- human CSF	- human CSF
8	Human 1875 NBH	Human 1875 NBH	- human CSF	- human CSF
9	Human 1875 NBH	Human 1875 NBH	- human CSF	- human CSF
10	Human 1875 NBH	Human 1875 NBH	ψ + human CSF	+ human CSF
11	* + human 1399	* + human 1399	+ human CSF	+ human CSF
12	ψ + human 1399	+ human 1399	+ human CSF	+ human CSF
13	+ human 1399	+ human 1399		
14	+ human 1399	+ human 1399		
15	* BSE 33	* BSE 33		ladder
16	ψBSE 33	BSE 33		
17	BSE 33	BSE 33		
18	BSE 33	BSE 33		

* samples were not treated with PK prior to WB assay

ψ samples were pre-treated with O2 aptamer post PK digestion but prior to electrophoresis.

BSL2 experiments were conducted using a similar protocol to above with the following exceptions. Samples were PK digested with 50 μg/ml at 45°C for 30 minutes (Thermomixer, Eppendorf) then combined with 3x loading buffer and PK deactivated by boiling for 5 minutes at 95°C. Samples were pre-treated with the P2O1 aptamer prior to loading in the gel. 12 μl of

samples was loaded into a 12 well gel (see Table 3.3 for outline) then electrophoresed at 110V for 10 minutes and 150V for 60 minutes. Three replicate gels of the samples were run, one was incubated with the P1O1 aptamer used in the above experiment, the second blot was incubated with P2O1 to test for epitope differences, and the third was incubated with HRP conjugated Bar224 Ab at 1:20,000 in Superblock.

Table 3.3. Western blot gel sample layout

Lane	**Gel 1 – P1O1 Aptamer**	**Gel 2 – Bar224 Antibody**	**Gel 2 – Bar224 Antibody**	**Gel 3 – P1O1 Aptamer**	**Gel 4 – Bar224**
1	*5037NBH	*5037NBH	*5037NBH	Ladder	Ladder
2	5037NBH	5037NBH	5037NBH	*TgA20NBH	*TgA20NBH
3	ψ5037NBH	ψ5037NBH	ψ5037NBH	TgA20NBH	TgA20NBH
4	*RML5	*RML5	*RML5	*5037NBH	*5037NBH
5	RML5	RML5	RML5	5037NBH	5037NBH
6	ψRML5	RML5	RML5	*eNBH	*eNBH
7	*E2	*E2	*E2	eNBH	eNBH
8	E2	E2	E2	*RML5	*RML5
9	ψE2	E2	E2	RML5	RML5
10	*E2P2	*E2P2	*E2P2	*E2	*E2
11	E2P2	E2P2	E2P2	E2	E2
12	ψE2P2	E2P2	E2P2	E2 rods	E2 rods

* samples were not treated with PK prior to WB assay

ψ samples were pre-treated with O2 aptamer post PK digestion but prior to electrophoresis.

RESULTS

Phase 1

We encountered extensive variability in the DNAse efficacy between batches. In one experiment with a particularly strong DNAse batch, we found identified a difference in digestion sensitivity between aptamers (Figure 3.4). We did not pursue this aspect further, but found it suggestive of tertiary conformation differences and stability of the different aptamers selected for this study.

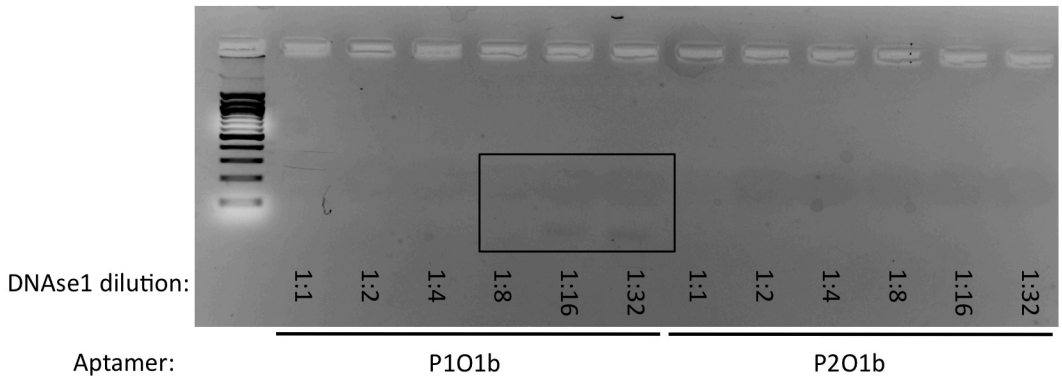
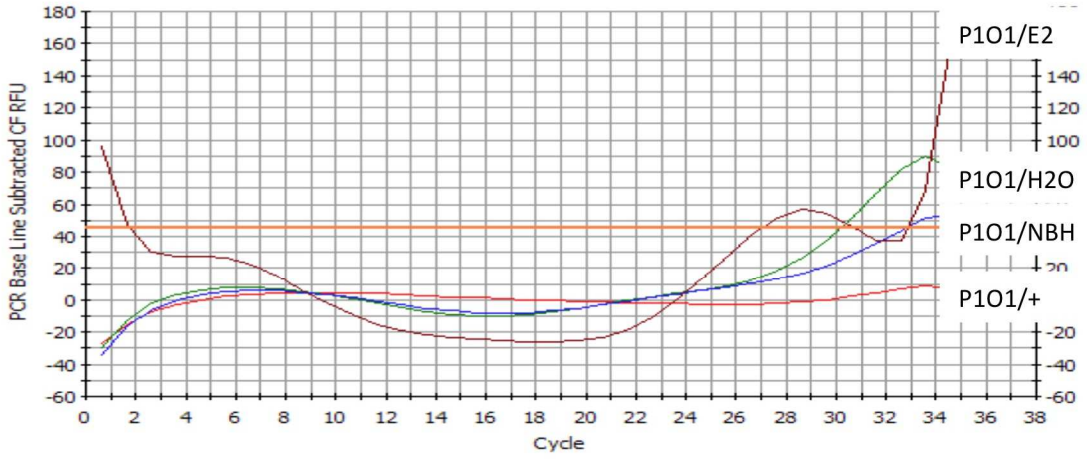


Figure 3.4. While testing for optimal DNAse digestion concentration we observed that there was a difference in DNAse sensitivity between aptamers.

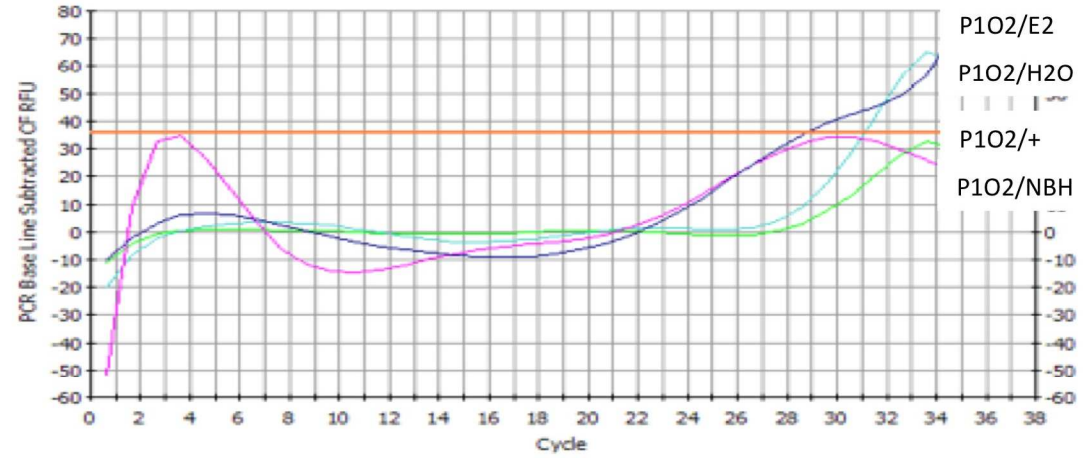
Testing the phase 1 aptamer sequences we found that P1O1 and P1O2 demonstrated specificity, but limited sensitivity, to the E2 PrP^{RES} while P1O3 recognized both PrP^{RES} and PrP^{C} (NBH; Figure 3.5 a-c). Modified phase 1 aptamers, P1O1b-P1O3b were more sensitive with all three specifically recognizing PrP^{RES}, but P1O3b again recognized PrP^{C}**.** P1O1b was the aptamer primarily used for subsequent experiments. We did not use P1O3 for any further experiments.

We also found that one of the control samples, aptamer diluted into water then treated with the standard DNAse and PK digests the other samples received, resulted in extensive false positives. This effect of water on amplification was consistent between samples and aptamers. We hypothesized that the aptamers might be dimerizing in the water and remained undigested by the DNAse and PK treatments.

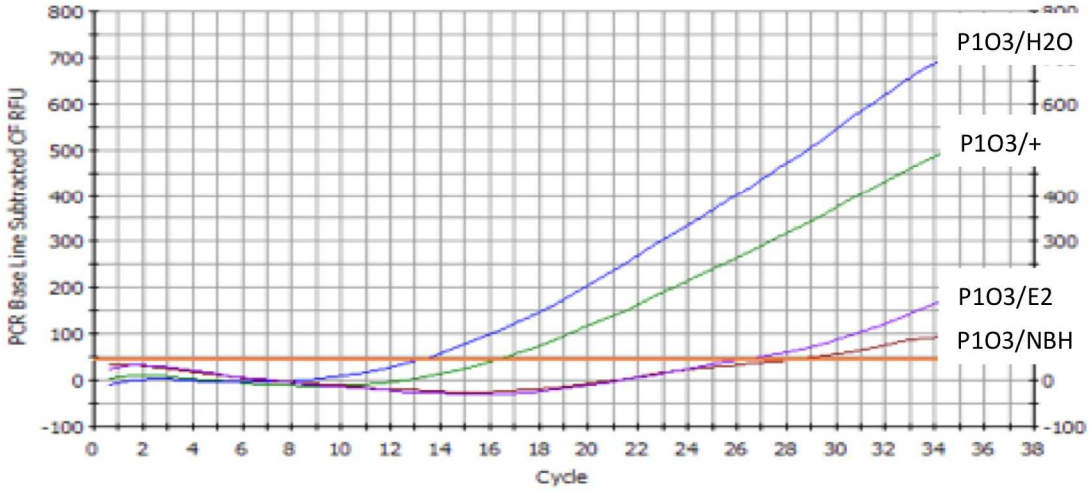
a.


PCR Base Line Subtracted CF RFU
Cycle
P1O1/E2
P1O1/H2O
P1O1/NBH
P1O1/+

b.


PCR Base Line Subtracted CF RFU
Cycle
P1O2/E2
P1O2/H2O
P1O2/+
P1O2/NBH

c.


PCR Base Line Subtracted CF RFU
Cycle
P1O3/H2O
P1O3/+
P1O3/E2
P1O3/NBH

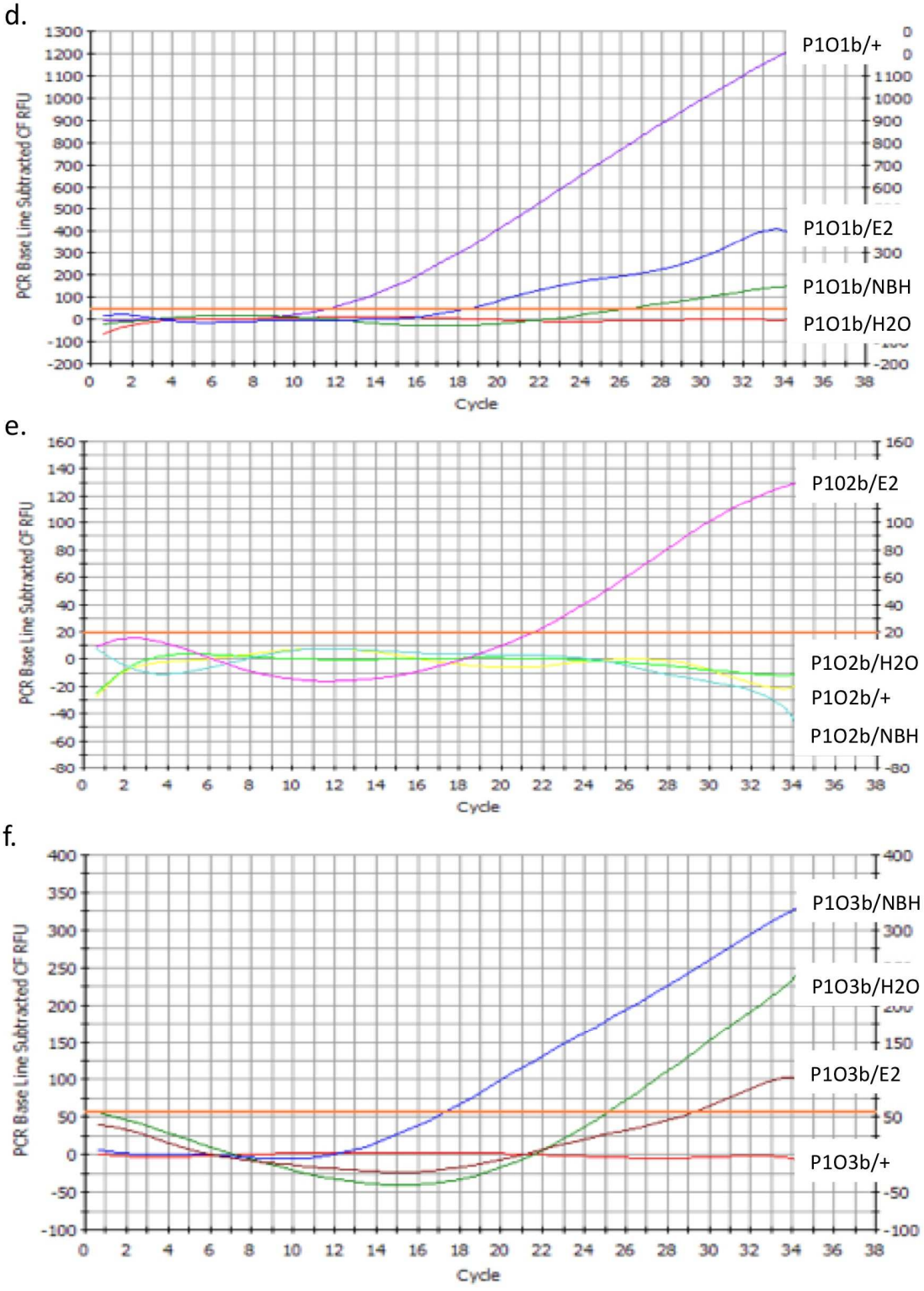


Figure 3.5. rtPCR of aptamers after binding to prion strain E2. Each panel contains a different aptamer with the following samples: 10^{-3} dilution of E2 prions, positive (+) control of 10^{-2} aptamer dilution, (-) control of DNAse digested aptamer in H2O, NBH (-) control. a. P1O1, b.

P1O2, c. P1O3, d. P1O1b, e. P1O2b, f. P1O3b. Panels d and f show P1O1b and P1O2b may be specific for E2 prions with little or no background.

After reselection in Phase 2 we found P2O1b to be the most successful of the aptamers from both phase 1 and 2, showing amplification in samples of all three CWD isolates, E1, E2, E3 out to 1:10 M. Aptamers were most effective on non-PK treated samples, but also worked on PK digested samples (Figure 3.6).

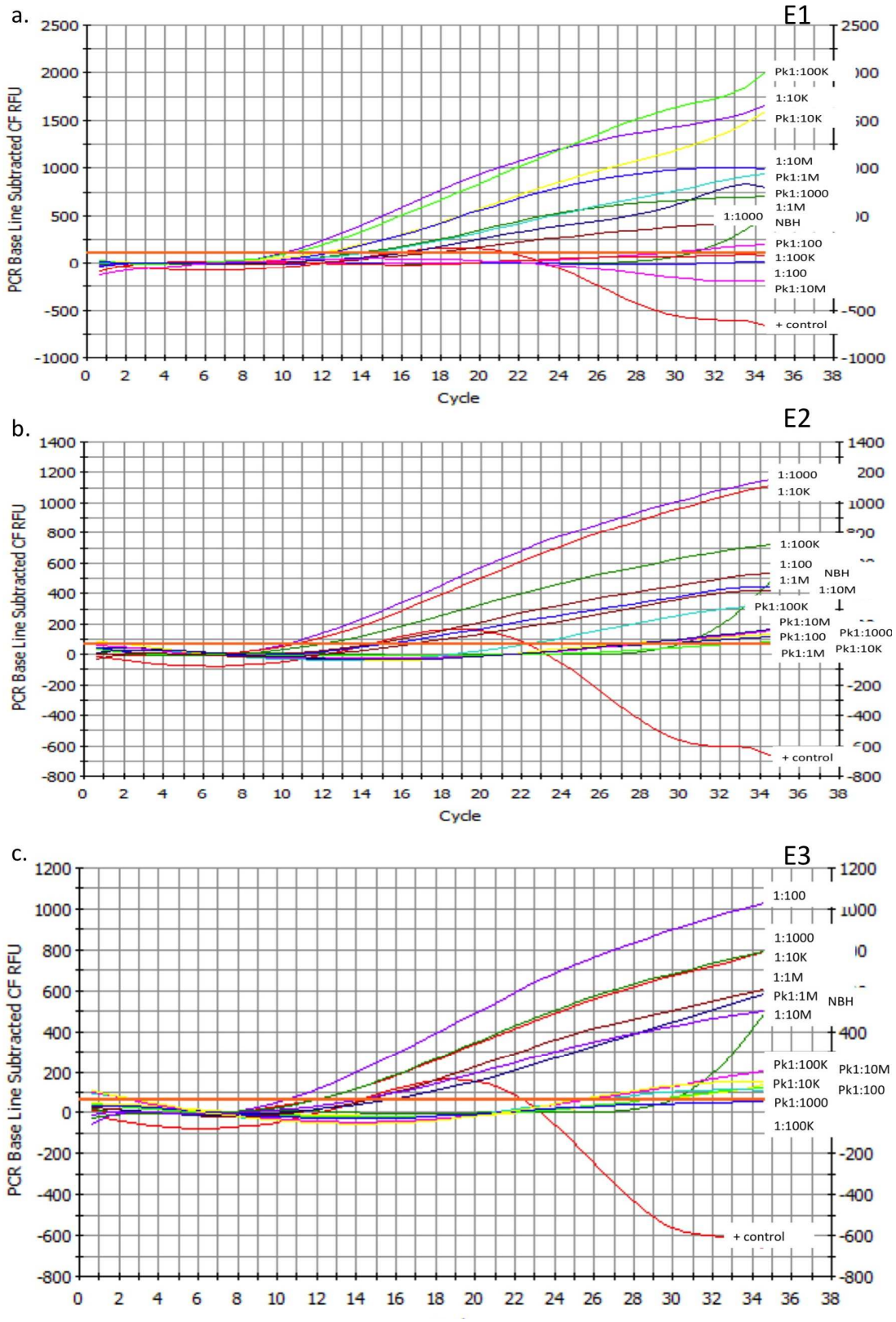


Figure 3.6. Aptamer P2O1b amplification by rtPCR for stains E1, E2, and E3 respectively. Each strain was serially diluted 1:10 and tested either undigested or PK digested.

Experiments testing serum with the two most promising aptamers, P2O1b and P1O1b were discouraging. Serum samples came from NHB mock inoculated TgA20 mice, and from RML inoculated TgA20 mice at 41 days post inoculation (dpi). The mock inoculated mice should have been negative, and the RML inoculated mice would have been early in infection, at about 20 days short of clinical disease. Both samples demonstrated non-specific binding to an unknown target protein in the serum (Figure 3.7).

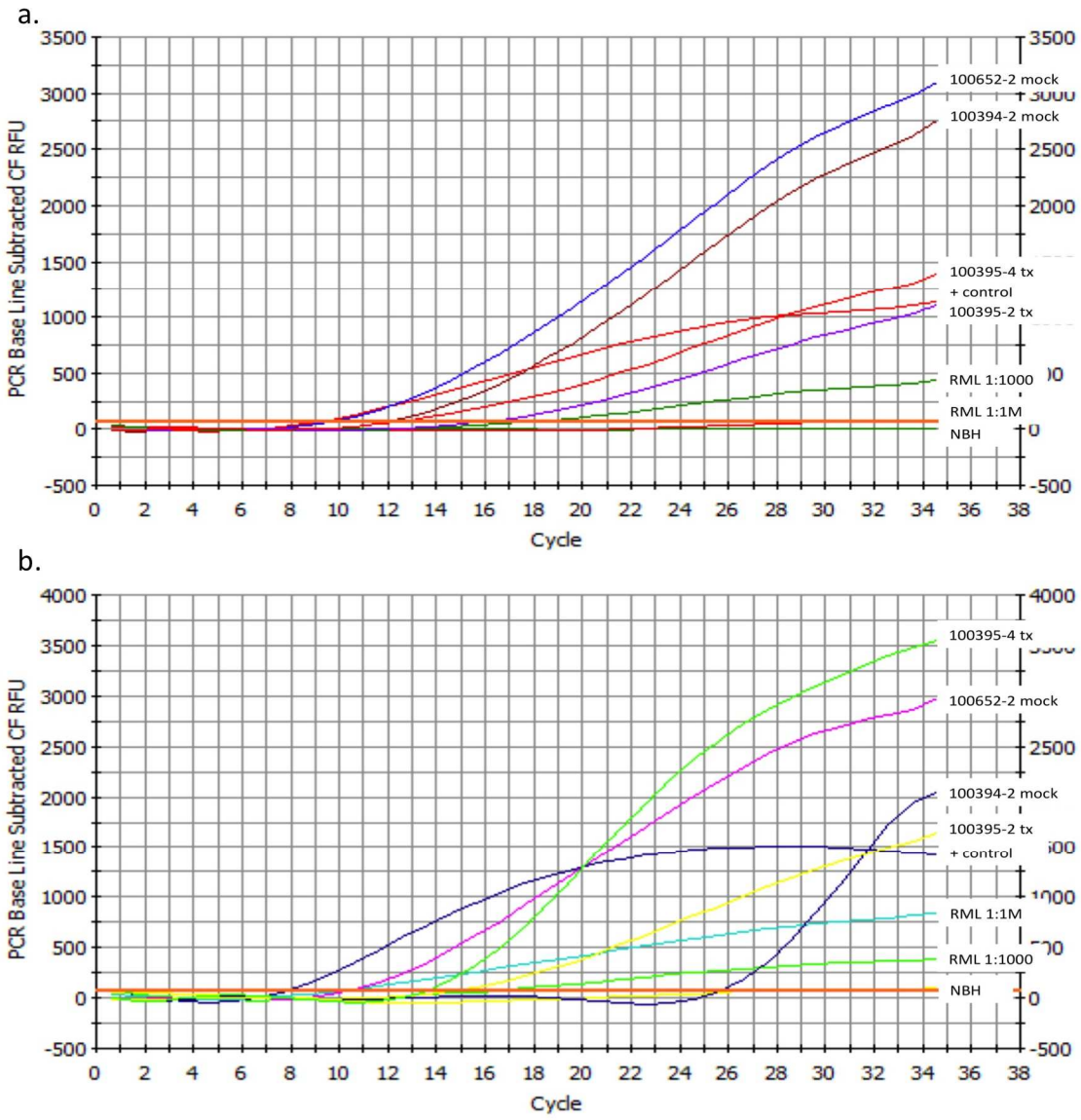


Figure 3.7. Preliminary results for two aptamers (P2O1b and P1O1b) used for detection of prions in mouse serum. Results indicate non-specific binding.

To remove the non-specific binding we tested a PK digest (10 μg/μl and 50 μg/μl at 45°C for 30 min). This digestion step did not mitigate the false positives. We also tried diluting the serum in H2O and NBH, which resulted in high levels of non-specific binding and little to no binding respectively with stronger signal in the negative controls than the test samples.

Elk feces appeared to have inhibitory factors that prevented any amplification. Dilution of the sample appeared to dilute out the inhibitory affect as well, which allowed for signal to be recovered, though it occurred in both positive and negative samples. Using this dilution strategy we tested two different fecal samples spiked with E2 (1:100 and 1:1000 E2 in solution) with P2O1b (Figure 3.8) and P1O1b. Both aptamers showed false positive signal in negative feces. This false positive effect was reproducible in fresh samples.

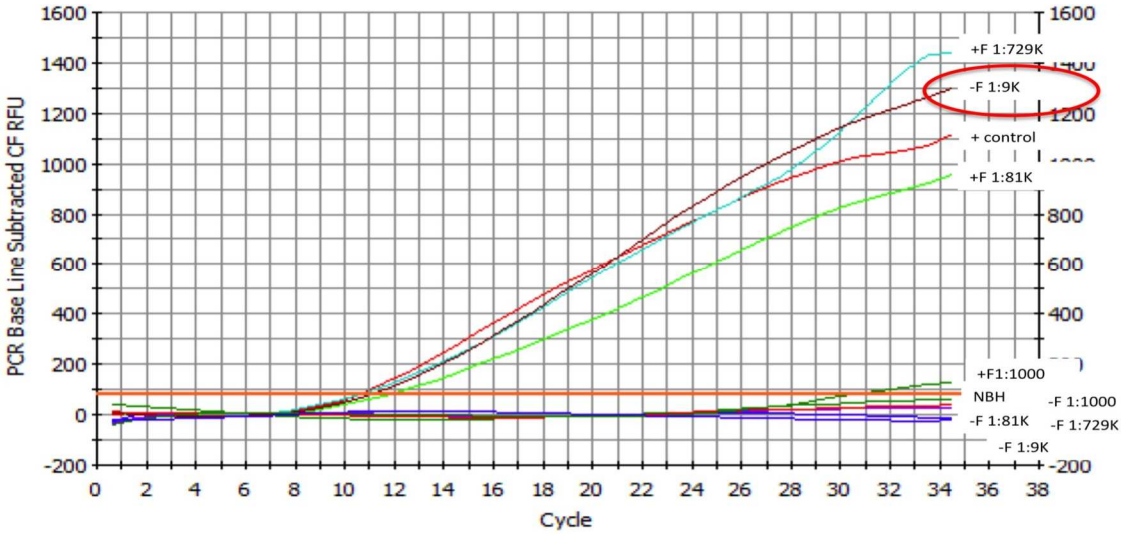


Figure 3.8. Representative results for: E2 spiked feces at 1:100 and 1:1000 concentrations, negative and positive spike samples were diluted out and tested with P2O1b. Amplified signal was detected in higher dilutions of spikes and negative controls.

Finally, spiked soil samples were tested to assess the efficacy of the aptamers in detecting prions in environmental samples. As was found for feces, dilution of soil improved results allowing for the dilution of perceived inhibitory factors that may be present in the soil. The first experiment resulted in successful amplification of spike samples with negative samples staying clean in the P1O1b aptamer run, while the P2O1b run had amplification in both positive and negative samples (Figure 3.9). Unfortunately, the results for P1O1b were not reproducible, with subsequent experiments resulting in false positives.

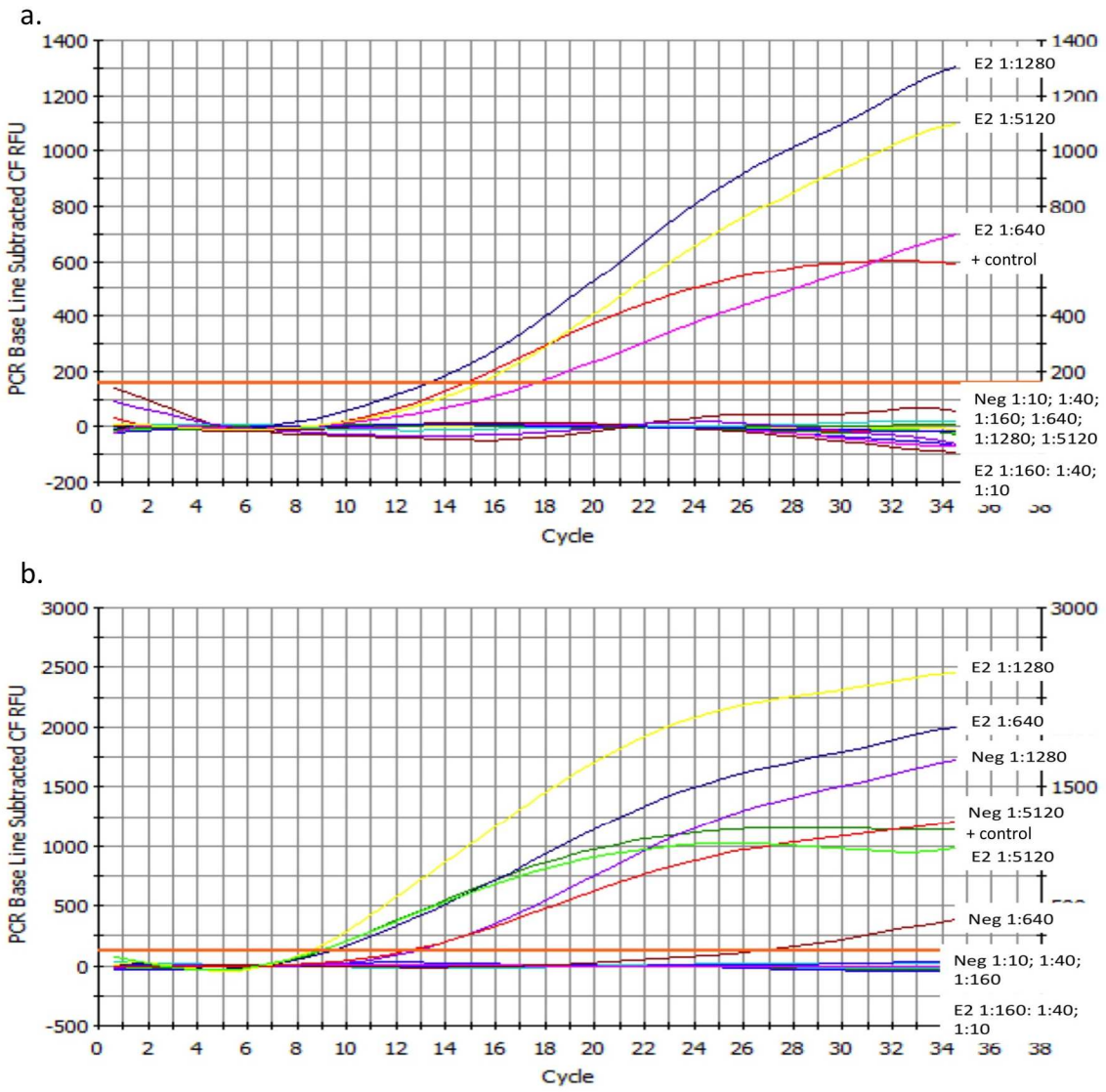


Figure 3.9. Preliminary results using P1O1b on soil showed promise, with all negative samples remaining below the threshold, and three of the higher dilutions showing amplification signal. This was unfortunately not reproducible. In the second set of samples with the P2O1b amplification signal was detected in negative soil dilutions suggesting non-specific binding to an unknown element in the soil.

A similar set of results was found for experiments on mouse urine, with inhibitory effects resulting in no amplification in urine from clinical mice. However, upon dilution of negative urine into water we again found false positive results as mentioned above (Figure 3.10). We did not try other diluents to see if the inhibitory affect could be overcome.

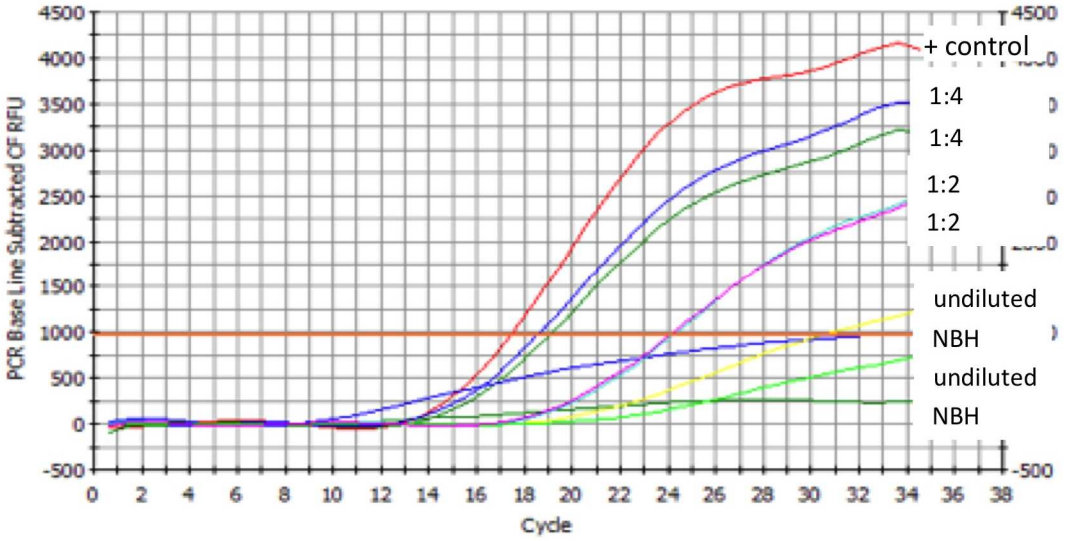


Figure 3.10. Uninfected 5037-mouse urine was diluted into DNAse and RNAse free H2O and treated with P2O1b. As the dilution increases so does the rate of false positives.

Late in the study, results from negative elk brain (eNBH) experiments cast doubt on all positive results and began to explain the high rate of false positives in negative samples. Samples of eNBH had nearly identical amplification profiles as E2 samples (Figure 3.11).

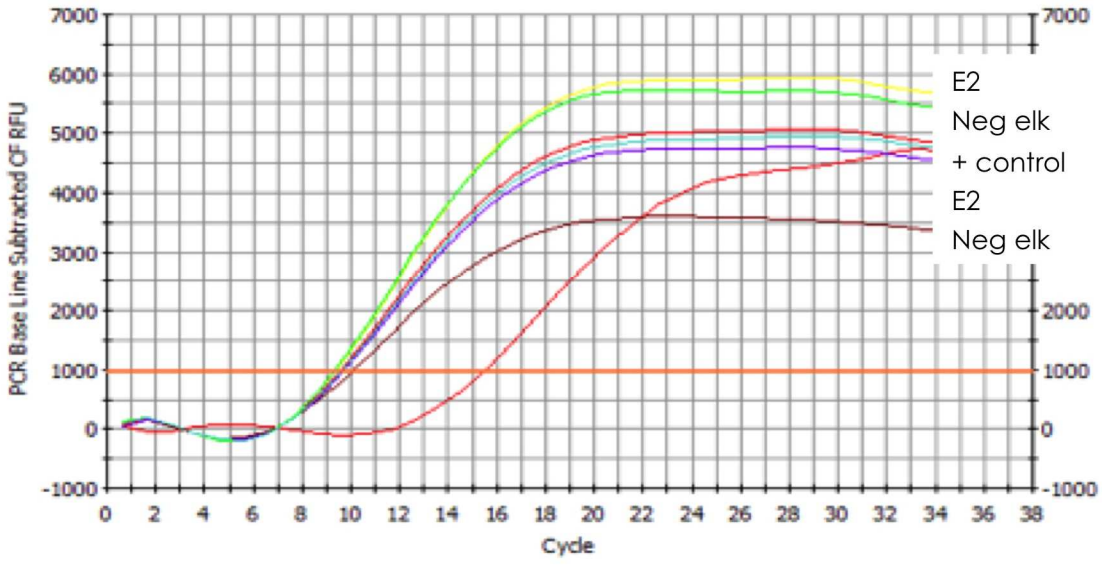


Figure 3.11. Original 10% stock of negative and positive (E2) elk brain homogenates have nearly identical PCR profiles when treated with P2O1b.

Dilution of eNBH into mouse NBH reduced the signal suggesting the false positive signal in elk tissues was diluted out by the mouse NBH (Figure 3.12). This was reproducible at dilutions tested up to 1:1,000,000

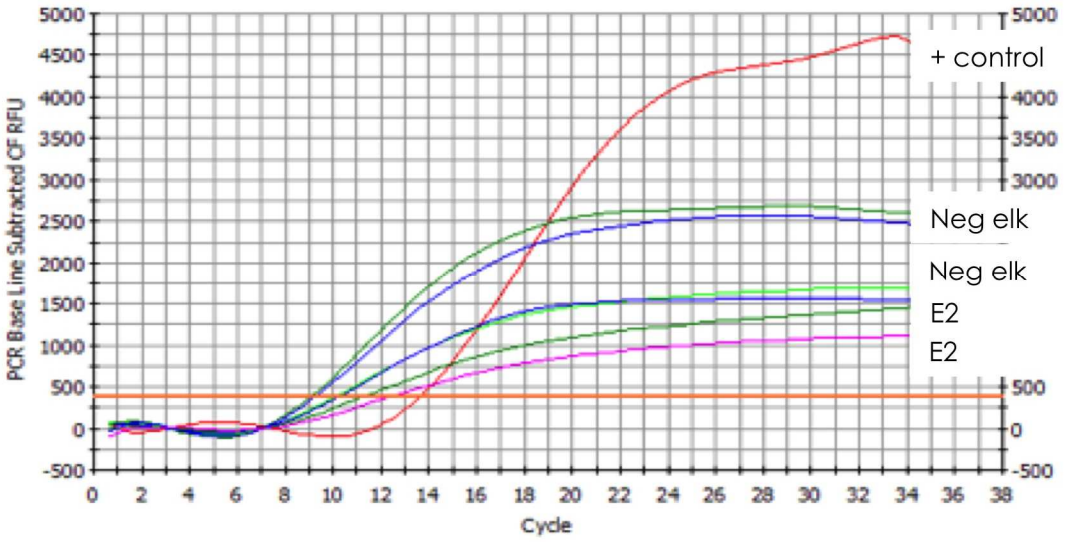


Figure 3.12. Dilution of positive and negative samples in to mouse NBH reduced the false positive signal when treated with P2O1b.

Pre-treatment of the brain homogenate with PK (Figure 3.13) digest did not reduce the false positive signal, indicating it is not a non-specific elk protein. Pre-treatment with DNAse at 2.5 units/µl was able to remove all amplification signal suggesting the non-specific interaction was an unidentified elk DNA molecule (Figure 3.14). There did seem to be a difference between

the two aptamers used, but neither was sufficiently specific to PrP^{RES}.

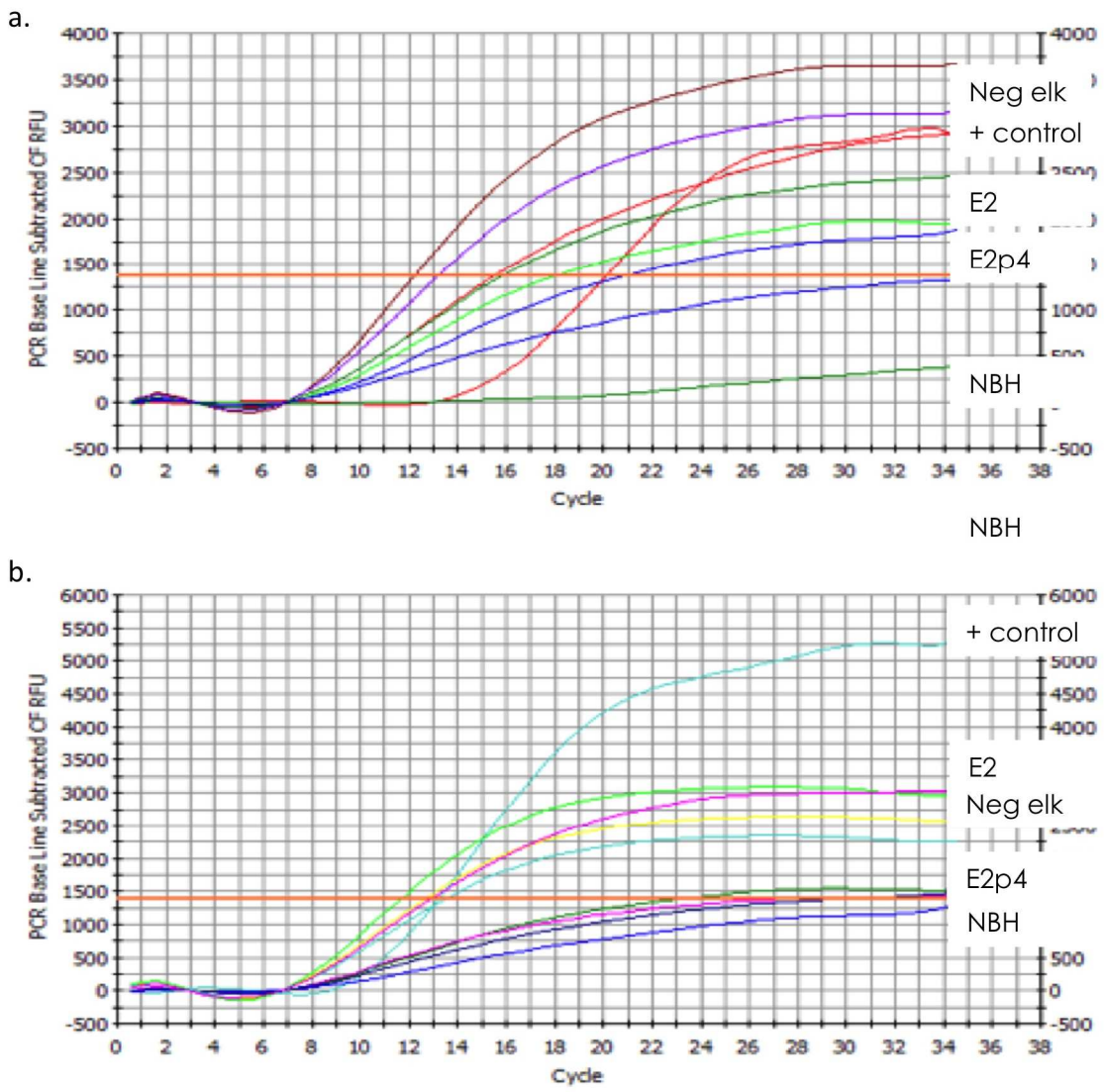


Figure 3.13. a. P2O1b with PK digested samples, **b.** P1O1b with PK digested samples. PK digestion did not reduce false positive rates in negative elk, positive elk or positive mouse brain homogenate. Differences were found between the two aptamers, P2O1b and P1O1b, however, both demonstrated false positives in negative elk tissue.

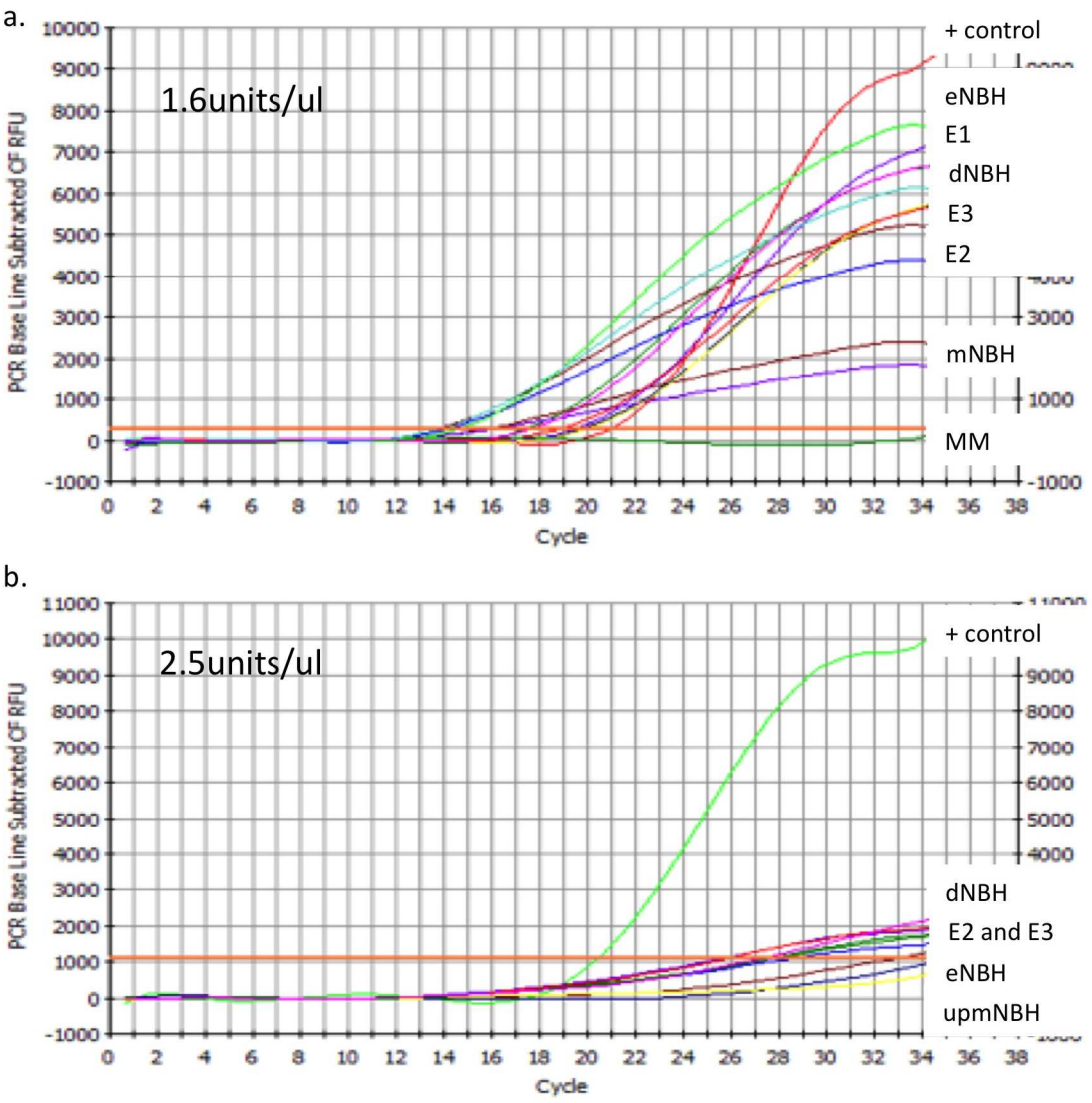


Figure 3.14. The stronger of the two DNAse digestions digested away the false positive signal resulting in no difference between spiked and negative samples when treated with P2O1b.

Superparamagnetic beads

In our preliminary runs using paramagnetic beads to try and enrich and better target the PrP^{RES} in the samples, we again encountered some type of inhibition in the samples contained the magnetic beads when placed in the 96 well plates and tested by rtPCR (Figure 3.15).

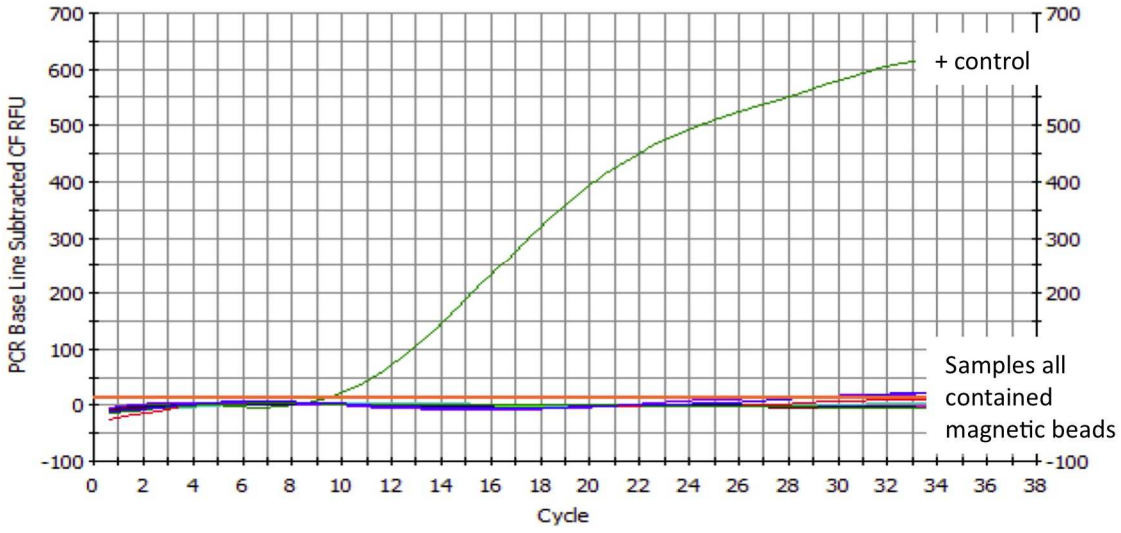


Figure 3.15. When the beads remained in the sample they seemed to inhibit the rtPCR reaction; the positive control P2O1b, which did not contain beads, was the only sample to show amplification.

The removal of the beads from the sample prior rtPCR resulted in successful amplification, as opposed to the complete suppression of amplification in the previous run. However, despite three washes of the beads with 1 x TBS there appeared to be sufficient residual aptamer in all aptamer treated samples to generate PCR amplification in all samples (Figure 3.16).

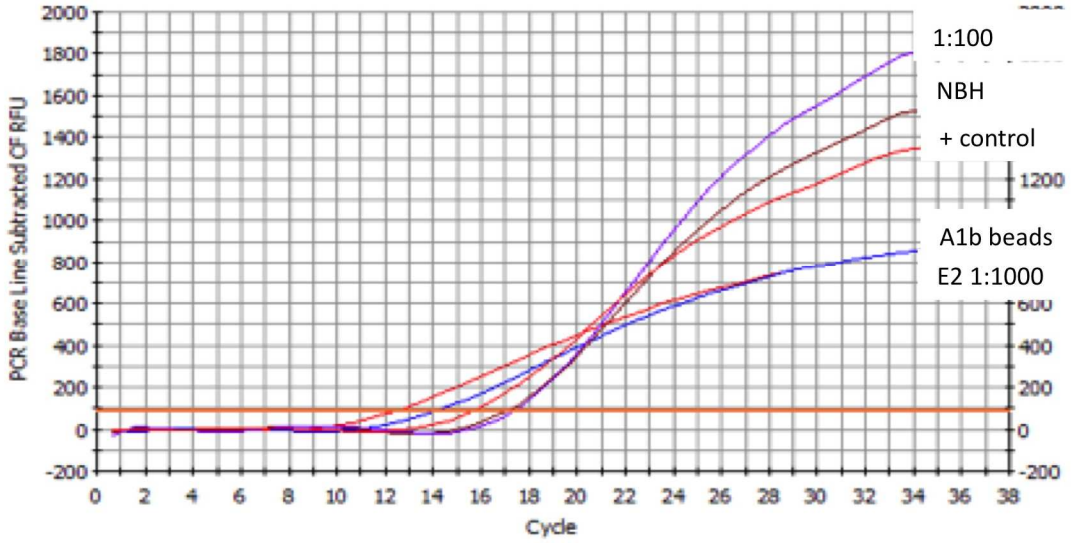


Figure 3.16. Despite three washes, samples contained excess aptamer and amplification was detected.

Results improved with a higher aptamer concentration of 100 pM (Figure 3.17). However, the negative controls of NBH and beads alone did still have amplification, though at later ct values allowing for differentiation from known positive samples. Additionally, the new washing protocol to remove unbound but incorporated aptamers may have reduced the level of false positives seen before.

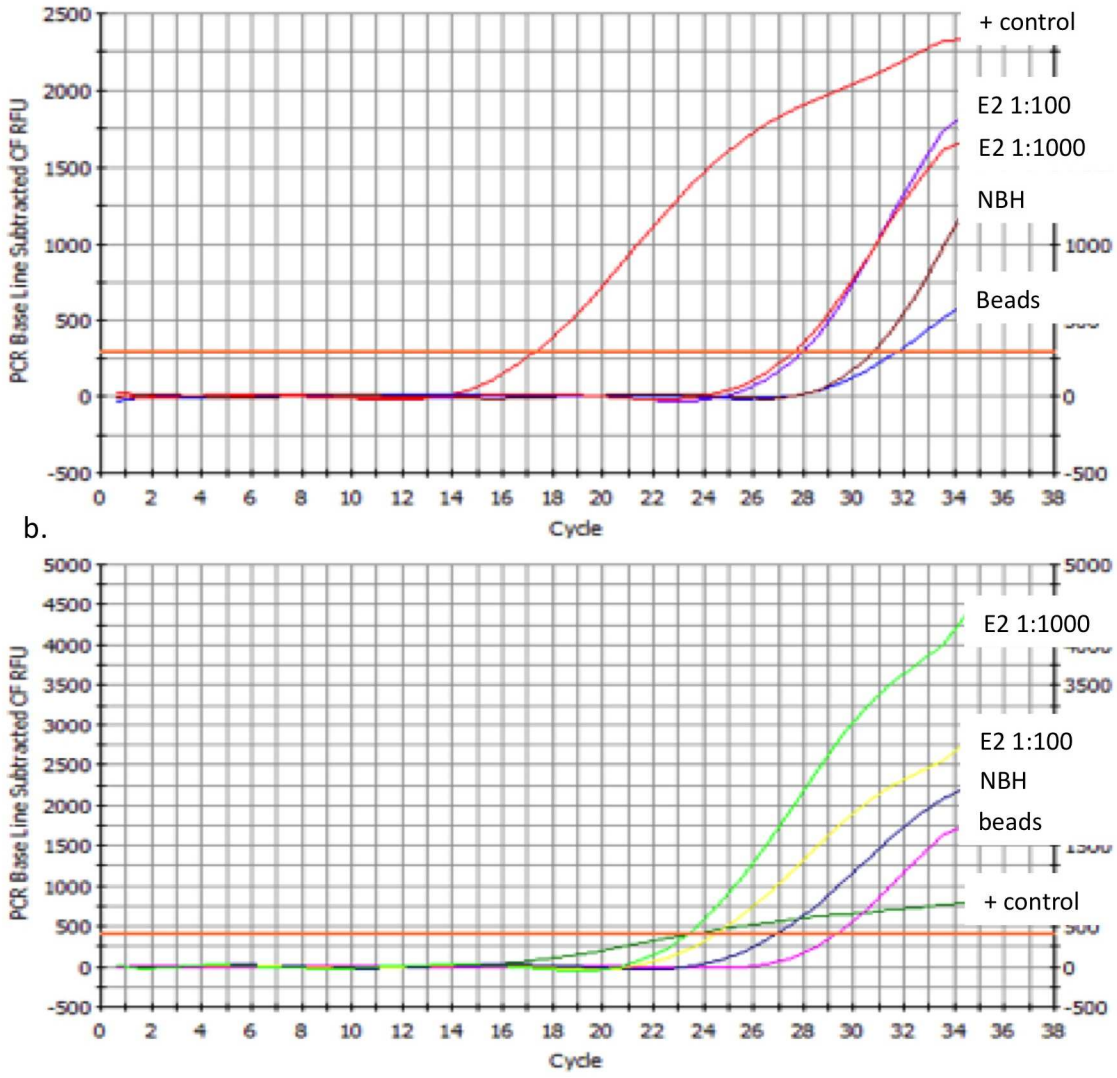


Figure 3.17. a. Improved results showing a distinction between positive and negative control samples when tested with the P2O1b aptamer. B. Again, the 100 pM concentration showed improved results with the negative controls coming up later.

Western blot – Case Western

Results from the Case Western BSL3 western blot experiment suggested that the P1O1 aptamer was specific for the misfolded, PK resistant portion of the brain homogenate (Figure 3.18). Specifically, the P1O1 aptamer recognized the vCJD equivalent tissues (TgHum BSE and human CJD; see orange arrows in lanes 4-7 and 11-14). The BSE (lanes 15-18 and 2-5) and

human CSF (second set of lanes, 9-12) samples are not as clear since the aptamer is recognizing material in the undigested lane but not the PK digested lanes. In Figure 3.19, the antibody blot indicates there may have been undigested material in lanes 1,2,3 and 8,9,10. In the aptamer treated blot it appears that the aptamer does not label the same undigested material suggesting it is specific for PrP^{RES}.

The pre-treatment of the positive samples with aptamer P2O1 appears to have retarded the mobility of the prions in the gel due to the biotinylated aptamer binding the prion. The signals at the end of those lanes are strong and are likely accumulations of excess unbound aptamer (see yellow arrow lanes 12 and 16, Figure 3.18.) Samples that did not receive a PK digest show more extensive banding patterns. It is currently unclear what those bands contain.

Overall, the aptamer treated blot showed bound PrP^{RES} with similar banding patterns to the antibody treated blot. Both blots showed positive material in BSE TgHum mouse and human CJD samples with strong signals. BSE and the other samples tested were less conclusive or negative.

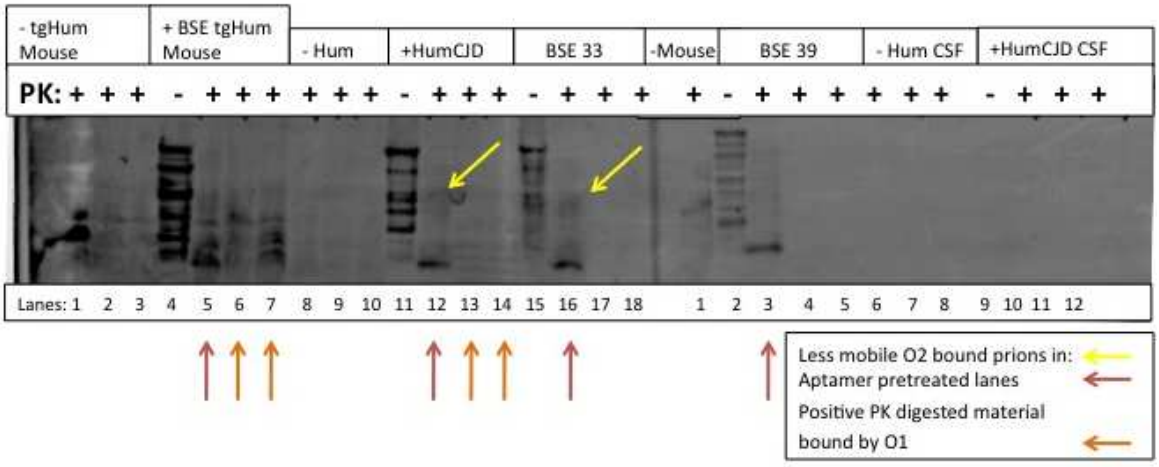


Figure 3.18. The above western blot shows results from the BSL3 aptamer P1O1 test suggesting the aptamer successfully recognizes PrP^{res}

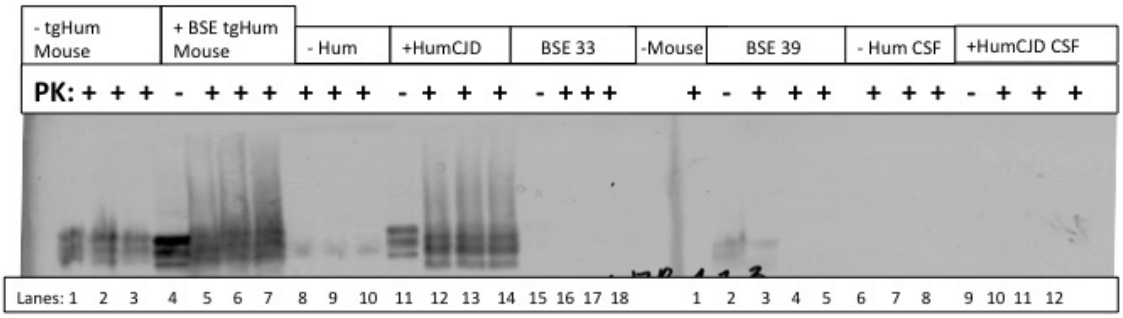


Figure 3.19. The above blot (Blot 2, Table 3.2) is the antibody (6H4, 8H4, 3F4) control blot verifying the presence of PrP^{C} and PrP^{RES}.

Western Blot – CSU

The replication of the above experiment using BSL2 material at CSU was less successful. Results did not show PrP^{RES} specificity for either aptamer on any of the PK digested samples but did recognize material in the undigested samples similar to the BSL3 experimental results (Figure 3.20 and 3.21). The antibody blot looked as expected (Figure 3.22) and did not seem to indicate a molecular weight shift of in the samples that were pre-treated with P2O1.

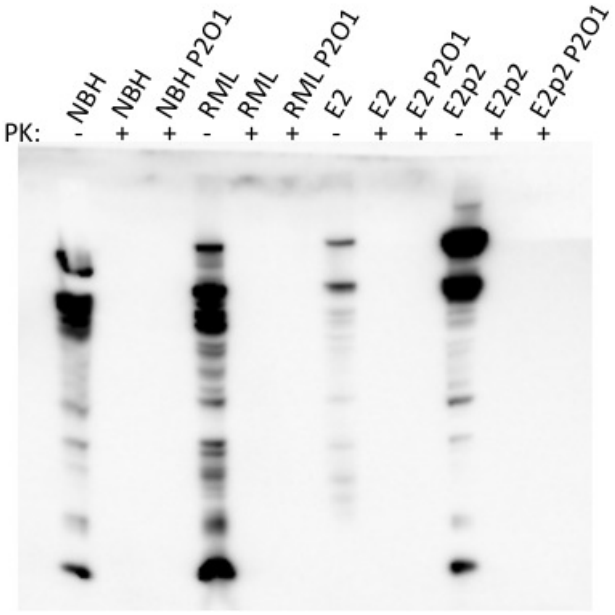


Figure 3.20. The P1O1 aptamer did not show specificity for PK resistant material, only undigested material.

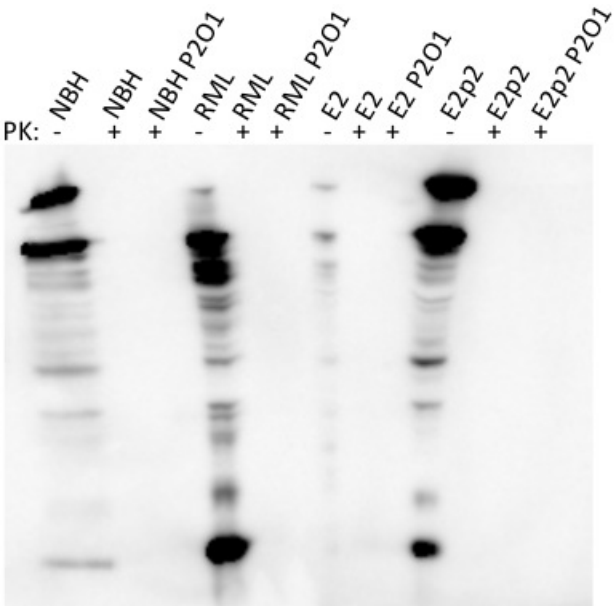


Figure 3.21. The P2O1 aptamer also did not show a binding specificity to PK resistant material, but did bind undigested material.

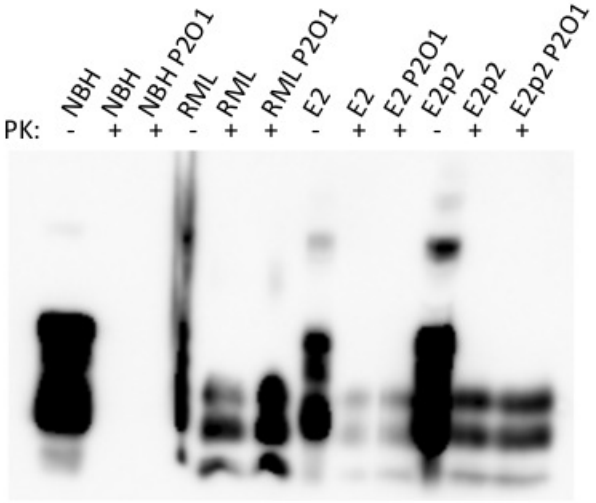


Figure 3.22. The control antibody Bar224 blot showed PK resistant material in digested lanes and no obvious shift in molecular weight for samples pre-treated with the P2O1 aptamer.

DISCUSSION

rtPCR

Experiments utilizing PCR to amplify aptamers tagged on PrP^{RES} were frustratingly inconsistent. Preliminary results suggested aptamer specificity for PrP^{RES} with and without PK digestion. However, often the specificity obtained in one experiment was difficult to reproduce.

Difficulties with DNAse efficacy also complicated experiments. The finding that the DNAse had different efficacies when used on different aptamers, as shown in Figure 3.4, was intriguing and supported the hypothesis that the aptamer sequence conveyed specific conformational and stability aspects through the tertiary structure. This was further demonstrated in the different binding properties of P1O1, P1O2 and P1O3 in Phase 1. The first two aptamers showed specificity for PrP^{RES} positive samples while P1O3 appeared to recognize both PrP^{RES} and PrP^{C}. This suggests the three aptamer sequences have affinity to different target epitopes, with P1O1 and P1O2 specifically binding to an epitope that is unique to the infectious prion protein.

An interesting artifact that was not sorted out was the false positive signal that occurred with the H_2O negative control. When combined with the aptamer, and treated with the standard DNAse and PK digestions, both tap water and dH_2O resulted in robust amplification signal, often above the positive control signal (Figure 3.5c.) We attempted to mitigate this false positive signal by optimizing the primers and aptamers tails to reduce the risk of primer dimmers, but it did not entirely resolve the issue. As a result we stopped using H2O as a control, under the premise that our positive control and negative brain homogenate sample would suffice.

Aptamers selected in the second phase, and given the modified tail sequence showed the most promise with positive recognition of all three CWD field isolates. P2O1b was able to recognize E1 with and without PK digestion, which is significant because the E1 CWD isolate

has a different PK cleavage product than E2 and E3, which prevents some antibodies from recognizing E1 after PK digestion (data not shown). P2O1b resulted in a strong amplification signal for E2 and E3 in undigested samples suggesting recognition of PrP^{RES} as well an unidentified non-specific interaction. This would also explain the amplification signal in NBH below undigested levels but equal to or above PK digested sample signals.

Serum samples from positive and negative sources all showed similar false positive signal, often significantly higher than the positive plate control. The PK treatment of serum to remove the many proteins present did not alter the false positive issue indicating the false positive signal was not a result of non-specific protein binding.

The opposite effect was found when testing feces, urine and soil. Preliminary experiments on all three sample types indicated the presence of inhibitory factors. This has been seen for feces and soil by other detection methods, particularly PMCA [20,25]. Pulford et al. were able to dilute out the inhibitory effect and maintain specific detection of PrP^{RES}, unfortunately that did not hold true for this assay. Diluting the spiked feces into NBH did allow for amplification at higher dilutions while lower dilutions (i.e., 1:100 or 1:300) did not allow amplification. Unfortunately, the same results occurred in the negative feces, indicating we had extensive non-specific binding.

As with feces, urine seemed to inhibit amplification. Dilution of the urine into water resulted in the false positives as was found previously when using water as a diluent. We did not pursue this sample type further, and instead moved to testing soil which has been a particularly challenging substrate to apply detection assays to [25-28]. Spike samples were successfully amplified using P1O1b, only after we diluted the soil sample. Once again, we were unable to replicate the control results, and routinely had false positive signal in the negative samples.

Interestingly we were using a sandy loam soil for our negative soil substrate, which has proven to be entirely inhibitory in PMCA [25]. Other soil types may behave differently in this assay, however, we did not pursue this avenue.

Late in the study we received some negative elk brain and included it in an experiment with aptamers. The eNBH was been confirmed negative by bioassay and PMCA so we were extremely surprise to see a false positive signal in the eNBH. The amplification profile was nearly identical to our undiluted CWD isolates suggesting the positive samples were also showing signal as a result of the non-specific interaction. Interestingly when we diluted the eNBH into 5037NBH as we had previously done with our CWD isolates when testing detection limits we were able to reduce the false positive signal. These results suggest that all of the positive signals in any elk tissues were a result of non-specific binding to an unknown target. PK treatment did not affect the amplification, indicating it was not a host protein interaction. The DNAse digestion on the other hand removed the signal across all elk samples, suggesting it could be host DNA that was binding the aptamer and resulting in rtPCR amplification. It is unclear if the aptamer was also binding PrP^{RES} in known positive samples. Our attempts at adapting the superparamagnetic beads for this assay were also fraught with difficulties and inability to reproduce results on positive and negative controls.

The aptamers seemed to possibly work on non-elk tissues with mouse NBH remaining negative in most experiments with higher levels of amplification in positive mouse tissues. Unfortunately, reproducibility of expected results was inconsistent and at this stage it is unclear why the negative samples would sometimes generate a PCR signal. Ideas for the cause of the false positives include: ineffective DNAse digestion of aptamers, contamination in the aptamer suspension, contamination in the primers, primer dimmers in dH_2O diluents, or contamination in

the samples. We did find that NBH could be used as a diluent instead of dH_2O. Inconsistencies between experiments, and clean NBH negative controls suggest that contamination is not likely, but cannot be ruled out.

Western blots

Results obtained on the BSL3 samples at Case Western were extremely promising, and actually might have been stronger had we not miscalculated the Tween detergent dilution (1% vs. 0.1%) for the aptamer and antibody incubations. However, we had clear binding by aptamers to CJD equivalent tissues such as +BSE TgHum mice and the human CJD brain homogenates. The aptamer also seemed to have limited to no affinity for negative samples that were partially PK digested, suggesting some level of specificity at the resolution of a western blot.

Unfortunately, despite the fact that the aptamers were selected against our E2 CWD isolate, there was no recognition of E2 when the western blot was probed by either aptamer P1O1 and P2O1. The aptamer bound to unknown material in the undigested samples, but in digested lanes there was no aptamer present in the sample. The reason for this difference as compared to the Case Western experiment is unknown and frustrating.

In summery, the positive amplification signal that is found in both positive and negative elk brain homogenate samples, but not in negative mouse homogenate samples, suggests the selected aptamers may have a non-specific affinity for elk specific molecules. The PK digest of the samples did not change the amplification profile of the samples indicating it is not a protein that is causing the false positives. Alternatively, the aptamer with it's primer-tails may be interacting with elk DNA resulting in recognition of similar host DNA specific to the elk but not found in mice. These results suggest that extensive negative selection must be conducted on a library of candidate aptamer sequences to prevent the types of false positive results we have been

reporting. Despite this setback, we believe this technology still holds promise for detecting infectious prion proteins in tissue samples, and that further research is warranted.

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CHAPTER 4:

INCREASED CWD PREVALENCE AND SUB-CLINICAL POPULATION IN FREE-RANGING ELK AS ESTIMATED BY PROTEIN MISFOLDING CYCLIC AMPLIFICATION

SUMMARY

Currently, prion protein immunohistochemistry (IHC) of brain tissue is the gold standard for CWD detection. However, this method may be insensitive to early or sub-clinical cases of CWD that may play an important role in disease transmission ecology. Alternatively, the serial protein misfolding amplification assay (sPMCA) is a reliable detection system that can assay many replicate samples for increased accuracy. This study compared the efficacy of IHC to sPMCA for the detection of low prion titers in naturally infected CWD-positive elk (*Cervus elaphus nelsoni*). By implementing a hierarchical Bayesian model, we estimated the specificity and sensitivity of all tests conditional on simultaneously estimated disease states. IHC test results were modeled as a Bernoulli trial while sPMCA test scores arose from a modified survival process across amplification cycles. Our results suggest that sPMCA of the obex is more sensitive (95%) in the detection of CWD prions than IHC of the obex (71%). Only through evaluation of multiple tissues does IHC sensitivity equal sPMCA. sPMCA can also return unbiased results in a shorter time frame than IHC, which requires more tissue, processing and time for microscopic evaluation. Prevalence estimates of CWD in this free-ranging population were estimated at 21.62% by sPMCA compared to a previously reported prevalence of 12.4% estimated by IHC. Our data show a previously unidentified sub-clinical prion-positive elk

population that could represent silent carriers and a source of prion shedding into the environment.

INTRODUCTION

Sensitive and accurate detection of Chronic Wasting Disease (CWD) prions is an essential component of the successful study and management of CWD in captive and free-ranging-wildlife. CWD is a neurodegenerative disease first seen in captive Colorado cervid populations in 1967, later identified as a transmissible spongiform encephalopathy (TSE) in 1978 [1] and first found in free-ranging populations in 1981 [2]. Prions, the putative infectious agent of TSEs, consist of a misfolded and insoluble aggregated form of the normal host-encoded prion protein (PrP^{C}) that is typically resistant to protease degradation and referred to as PrP^{RES} or PrP^{CWD} for CWD. CWD is the only known TSE to occur in free-ranging populations and affects several cervid species including elk (*Cervis elaphus nelsoni*), mule deer (*Odocoileus hemionus)*, white-tailed deer (*O. virginianus*) and less commonly moose (*Alces alces spp.*). Prevalence of CWD in free-ranging cervid populations in Colorado and Wyoming ranges from 0-30%, though prevalence in elk herds is generally lower between 0-3% in Colorado [3-6].

It appears that of known TSEs, CWD and scrapie (a TSE of sheep) are capable of both horizontal transmission from infected individuals as well as efficient indirect transmission from contaminated environments [7-14]. Experimental and anecdotal evidence suggest that CWD and scrapie are resistant to environmental degradation and remain infectious for years [9,11,15]. Despite a cross-species transmission capability of CWD within cervids, the pathogenesis of the disease is different between deer and elk. In deer, studies suggest that the prion infects and replicates in peripheral lymphatic tissues prior to neuroinvasion [16]. Previous studies suggest that elk demonstrate a different pathogenesis with the prion infection first detectable in the obex,

and later disseminated throughout lymphatic tissues [17-20]. It is unclear if route of inoculation or prion strain can also affect these apparent differences.

Infected animals are known to shed prions into the environment through saliva, feces, urine and even antler velvet [21-25]. Studies have successfully transmitted CWD through a single dose of urine or feces from a clinically infected animal indicating that sufficient prions are shed to the level of an infectious dose at the time of clinical disease [21,26]. What remains unclear, however, is at what stage in the disease course the animal begins to shed prions into the environment. If shedding occurs early in disease, a sub-clinical animal could not only be shedding prions into the environment increasing the infectious reservoir, but could also be horizontally transmitting disease. Unfortunately little is known about the prevalence of early disease, or a sub-clinical population, and what role in the transmission ecology that population might play.

The PRNP genotype of the host is known to influence the susceptibility and disease course of CWD. Specifically for elk, a polymorphism at allele 132(M/L) can dramatically affect the susceptibility of inoculated elk [27]. Experimental and observational evidence suggests that 132LL elk are rare ($\leq$ 2.5%) in free-ranging populations [28,29], and when inoculated 132LL elk have a dramatically delayed disease course as compared to MM and ML elk [27,30]. It remains to be shown if genotype affects transmission and shedding dynamics of prions. In addition to a genetic effect, population studies have also demonstrated an age effect on prevalence, with increased rates of CWD in elk between 2-11 years [31,32].

Currently, prion protein immunohistochemistry (IHC) of brain tissue and lymph nodes is the gold standard for CWD detection (APHIS 8/8/13, http://www.aphis.usda.gov/animal_health/animal_diseases/cwd/diagnostics.shtml). However,

this method requires 2-3 weeks for sample preparation, relatively large quantities of well-preserved tissue and specialized training for accurate microscopy work. Alternatively, the serial protein misfolding cyclic amplification (sPMCA) assay has emerged in the field of prion research as a reliable and sensitive detection assay for a variety of tissue and sample types [23,24,33-35]. Haley et. al. [36] compared sensitivity of sPMCA vs. IHC in longitudinal tonsil biopsies from experimentally inoculated whitetail deer. Findings from that study also indicated an abbreviated sPMCA protocol applied to lymphatic tissue of deer demonstrated a genotype-dependent sensitivity in CWD detection as compared to IHC.

Our lab has developed a more sensitive sPMCA protocol allowing for maximal amplification optimizing both sensitivity and specificity in less than two weeks. Our study looked at naturally infected elk from a free-ranging Rocky Mountain National Park (RMNP) herd. We used sPMCA to test obex tissues, the primary site of early infection in elk. Here we discuss our findings of improved sensitivity to early preclinical or sub-clinical cases of naturally infected CWD-positive elk (*Cervus canadensis nelsoni*), by sPMCA assay as compared to the efficacy of traditional IHC microscopy.

METHODS

Elk brain tissue samples

Brain tissues were collected at necropsy from free-ranging elk in RMNP in collaboration with a RMNP elk study [37]. Briefly, elk were initially captured, sampled and collared in 2008 (n=136). Rectoanal mucosal-associated lymphatic tissues (RAMALT) samples were collected on each elk during initial capture and tested for CWD by IHC [38]. In 2008 the animals that were identified as CWD-positive (n=13) were recaptured, euthanized and necropsied within two months of original capture. In subsequent years 20, 25 and 34 animals were recaptured,

euthanized and necropsied. Elk were immobilized and euthanized in the field [37] then transported to the TSE necropsy facility at the Colorado State University Veterinary Teaching Hospital within 8 hours of euthanasia. Field euthanasia and subsequent necropsies were approved by NPS (permit ROMO-2007-SCI-0077), Colorado Division of Wildlife (permit TR1081), and CSU IACUC (permit 07-231A).

Multiple tissues were collected from each animal. This study compares IHC results from the lymph nodes (palatine tonsil, retropharyngeal medial lymph node, submandibular lymph node and RAMALT) and obex (region of the brain stem at the level medulla oblongata [39]) to sPMCA results from the obex alone. Lymph nodes and obex were collected during necropsy [37]. All lymph node samples and half the obex sample fixed for IHC, the other half of the obex was stored in a whirl pack at -80° C for testing by sPMCA.

IHC

Tissues including sections of palatine tonsil, retropharyngeal lymph node (RPLN), RAMALT, and obex were examined by immunohistochemistry as previously described [38]. Briefly, tissues were fixed in 10% neutral buffered formalin, prepared on slides, immunolabeled with anti-prion protein monoclonal antibodies (mAbs) F99/97.6.1 (mAb 99) and mAb P4. PrP^{CWD} was identified by the presence of red aggregate deposits in neural and lymphoid tissues. A scoring system was used to evaluate intensity, 0-10, of PrP^{CWD} staining in the obex as outlined in [40].

sPMCA and western blotting

Frozen obex samples were slightly thawed and approximately 200 mg of sample was collected from the interior of the obex sample, placed into a 2 ml tube containing silica beads and 180 μl of sPMCA buffer #1 (150 mM NaCl, 4 mM EDTA, in PBS) was added. Tissues were

homogenized using a FastPrep machine (Thermo Scientific) as outlined in [41]. The clarified 10% homogenate supernatant was removed and stored at -80°C.

sPMCA amplification substrate consisted of 10% normal brain homogenate (NBH) prepared in a prion-free room from Tg5037 mice expressing cervid PrP^C as previously described [41]. Twenty-five micro liters of RMNP elk obex homogenate was added to 25 µl NBH in 0.2 ml tubes. Samples were sonicated in the Misonix 4000 sonicator horn (Qsonica Inc., Farmingdale, NY) for 40s every 30 minutes for 24 hours at 37°C constituting one round as previously described [24]. For each subsequent round, 25 µl of each sample from the previous round was combined with 25 µl of NBH. To balance desired sensitivity (>80%) and specificity (>99%) [35] duplicate samples were run for a total of 6 sPMCA rounds, a total of 3-6 replicates were run for each elk sample. Each sPMCA experiment contained at minimum six NBH-negative controls and two positive plate controls (CWD-positive elk brain homogenate E2, 1:1000). The last year of samples were also run with a minimum of six negative free-range elk brain homogenate samples (eNBH). The negative elk brain was collected from a female elk in Montana, a state, which remains CWD-free at the time of this study, and homogenized, as previously described. The eNBH was also confirmed negative by bioassay in CWD susceptible mice (data not shown). All 2010 and 2011 samples were run blinded.

Positive samples were identified by western blot as previously described [41]. Briefly, 18 µl of sample was digested with 2 µl of 50 µg/ml proteinase K (PK, Roche, Basel, Switzerland) for 30 minutes at 45°C. Samples were electrophoresed, electro-transferred to PVDF membranes and visualized with HRP-conjugated anti-PrP Bar-224 monoclonal antibody (SPI bio). If samples were found positive by sPMCA the replicate was given a score as outlined in Pulford et. al. [24]. Briefly, if a sample that came up in the second round, it would also be

positive for the subsequent rounds and would receive a sample score of 5, a sample that showed positivity in the last round would only receive a score of 1 and a negative sample would be scored 0.

Management of sample cross-contamination

The sensitivity of sPMCA raises concerns of cross-contamination between animal samples both at the necropsy and in the laboratory. During the first three years of necropsies, decontamination was not standard practice during tissue collection due to logistical difficulties. Necropsies in 2009 were performed in a new TSE necropsy room, reducing the risk of contamination. At the start of the 2011 necropsies, a decontamination protocol was put into place in the TSE necropsy room to further prevent cross-contamination during sample collection. Measures included SDS AcOH [42] treatment of working surfaces and all necropsy instruments, glove changes and apron changes between animals. Additionally, disposable sterile scalpels were used for all CNS tissue harvest. Sample preparation in the laboratory used protocols to prevent cross-contamination at the lab bench, including sterile scalpels, forceps and clean gloves used on each sample during sub-sampling. Similarly, during homogenization and sPMCA, samples were handled with clean gloves and treated as though positive.

Prevalence, Sensitivity and Specificity Estimates

Specificity, sensitivity and disease prevalence were estimated by hierarchical Bayesian analysis. Traditional specificity and sensitivity calculations using the 2x2 square method cannot be applied here because IHC, the current gold standard is considered perfect and thereby cannot be used to estimate sensitivity and specificity of sPMCA. Instead Bayesian analysis needs to be applied to detect the true imperfections in the gold standard, and allow for comparison to sPMCA. Additionally, the use of hierarchical models acknowledges and allows for imperfect

data and systems as opposed to other modeling techniques that require the assumption of error-free data and a perfect system. This flexibility of hierarchical models allows us to use samples collected all four years but still account for the possibility of contamination in two of those years (2009 and 2010). Additionally, hierarchical Bayesian modeling gives us probability estimates and distributions of the parameter estimates, such as disease prevalence.

The model is represented as a network diagram in Figure 4.1. Each animal was considered to have a true latent disease state, denoted z_i, where $z_i = 0$ when animal i is healthy and $z_i = 1$ for a CWD-positive animal. Disease state was modeled such that $z_i \sim Binom(\pi_i)$ where $logit(\pi_i) = \beta_0 + \beta_1 * age_i^2 + \beta_2 * age_i^2$ to reflect the well-understood correlation between disease and age [31,32]. All tests were modeled as binomial responses given the latent disease state of the animal and the test performance statistics of specificity and sensitivity. Each IHC tissue was modeled as independent given the disease state of the sample. For example, the IHC results for the obex tissue was modeled as follows:

$$y_{obex,i} \sim \begin{cases} Binom(1 - Spec_{IHC}), & z_i = 0 \\ Binom(Sens_{obex}), & z_i = 1 \end{cases}$$

where the specificity is the probability of a true-negative, $P(test = 0|z = 0)$, and sensitivity is the probability of a true-positive, $P(test = 1|z = 1)$. Each IHC tissue was modeled as an independent response given the sensitivity for that tissue but all IHC tests shared a specificity that was assumed to equal 1. This represents the expert opinion that in qualified hands obex and lymph node IHC analysis does not return false-positives (APHIS 8/8/13, http://www.aphis.usda.gov/animal_health/animal_diseases/cwd/diagnostics.shtml). The sPMCA test arises from a different process and as such is modeled to reflect that. All samples were subjected to six rounds of amplification and tested for the presence of PrP^{CWD} at each cycle. We

denote sPMCA results for individual *i* and replicate *j* across amplification rounds *t = 1:6* as $w_{i,j,t-1}$ which is contingent upon the latent disease state of individual *i*, Sensitivity and Specificity probabilities, *Se* and *Sp*, and the result from the previous round where applicable, $w_{i,j,t-1}$.

$$w_{i,j,1} \sim \begin{cases} Binom(1 - Sp_1), & z_i = 0 \\ Binom(Se_1), & z_i = 1 \end{cases}$$

$$w_{i,j,t} \begin{cases} \begin{cases} \sim Binom(1 - Sp_t), & z_i = 0 \\ \sim Binom(Se_t), & z_i = 1 \end{cases}, & w_{i,j,t-1} = 0 \\ = 1, & w_{i,j,t-1} = 1 \end{cases}$$

Se and *Sp* then become a vector of probabilities that represent the probability of a sample transitioning from a negative test result to a positive at each amplification round. These probabilities are modeled as flat Dirichlet distributions of length $T+1$. The final element in these vectors represents the probability of a negative test result so that the probabilities sum to one. The sPMCA part of the model is a modification of the Cormack-Jolly-Seber survival model with perfect detection. The parallel occurs because once a sPMCA cycle becomes positive, the following rounds will also be positive, much as a mortality event at any time guarantees all following times maintain that state. The model was fit to the data in JAGS 3.1.0 (Plummer, 2003, 2011a) with the rjags package (Plummer, 2011b) in the R 2.15.1 computing environment (R Development Core Team, 2012).

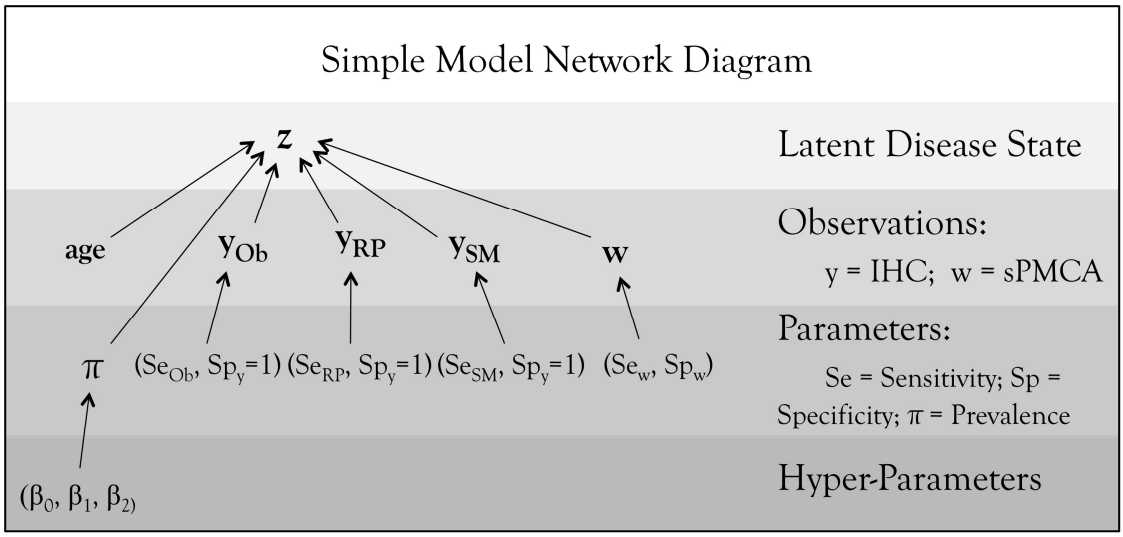

Figure 4.1. Network diagram outlining the model used to estimate specificity and sensitivity of IHC and sPMCA and population prevalence of CWD. Obex (Ob), RPLN (RP), SMLN (SM).

Errors in specificity, false-positives, occurred as a result of cross-contamination of the sample during necropsy or possibly by spontaneous misfolding during sPMCA. We previously reported our method of sPMCA has a specificity of 99.59% in the laboratory setting [35]. Negative samples used for this sPMCA experiment were used to show specificity in our laboratory setting, but do not account for possible necropsy contamination of the elk tissues. To remove bias from possible necropsy related false-positives in years 2009 and 2010 we separated "Trusted" from "Unknown" samples. Trusted samples are those found positive by IHC and all samples from 2011. Unknown samples are IHC-negative samples, which were positive by sPMCA. These samples could be true, sub-clinical positives outside of the detection limit of IHC, or they could be false-positives resulting from contamination during sample collection at necropsy. To maintain a conservative estimate of the specificity of sPMCA, Trusted and Unknown samples were analyzed separately.

Errors in sensitivity, or false-negatives, for either assay occurred for two reasons, the concentration of PrP^{CWD} was beyond the detection limit of the assay or despite the presence of

PrP^{CWD} in the tissue, the exact portion assayed did not contain detectable levels of PrP^{RES} [37,43]. All estimates are reported with a 95% Bayesian credible interval (CI).

RESULTS

Of the 85 elk tested in this study, 21 animals were IHC-positive in one or more tissues (Table 4.1). Of those IHC-positive animals, sPMCA identified 20 correlating obex samples as positive. The one sample that sPMCA did not generate a positive result for was a 2011 sample found to be IHC-positive in the RPLN only. sPMCA did identify an additional 18 IHC-negative samples as PrP^{CWD}-positive. A high rate of sPMCA-positive samples in 2010 suggests an unidentified portion of those samples may be false-positives. This may also be the case for 2009. However, sPMCA did identify 4 additional positives in 2011 compared to the three found by IHC (one of the IHC positive samples was the single disagreement mentioned above). These findings in 2011 we consider reliable due to the effective decontamination protocol applied in 2011.

Table 4.1. Summary of detection assay results

Sampling Year	***n* = elk sampled**	**sPMCA + (%)**	**IHC + (%)**				
			Obex	*RPLN*	*Ob & RP*[b]	*SMLN*	**IHC+ Total**
2008[a]	11	11 (100)			11		11 (100)
2009	17	5 (29.41)			1	1	2 (11.76)
2010	24	17 (70.83)[c]	1	3	1		5 (20.83)
2011[d]	33	6 (18.18)		1	2		3 (9.09)
Totals	85	39 (45.88)					21 (24.7)

[a] all animals were RAMALT-positive at start of study, euthanized in the field within 2 months and sampled further

[b] PrP^{CWD} was found in both obex and RPLN samples

[c] Cross-contamination of tissue samples at necropsy likely resulted in false sPMCA-positives this year

[d] A decontamination protocol was put in place to prevent cross-contamination at necropsy

We found a strong correlation between sPMCA and IHC scores for each elk sample (Figure 4.2). Linear regression found a positive association (slope=0.39, R^2=0.64) between IHC and sPMCA samples in agreement. Samples that disagreed, "Unknown samples", were not included in the linear regression, but are overlaid in Figure 4.2 to show the low sPMCA scores of samples that were otherwise IHC-negative. This correlates with the low amounts of PrP^{CWD} predicted to exist in early and sub-clinical, disease. Each data point in Figure 4.2 is the mean of replicates per animal.

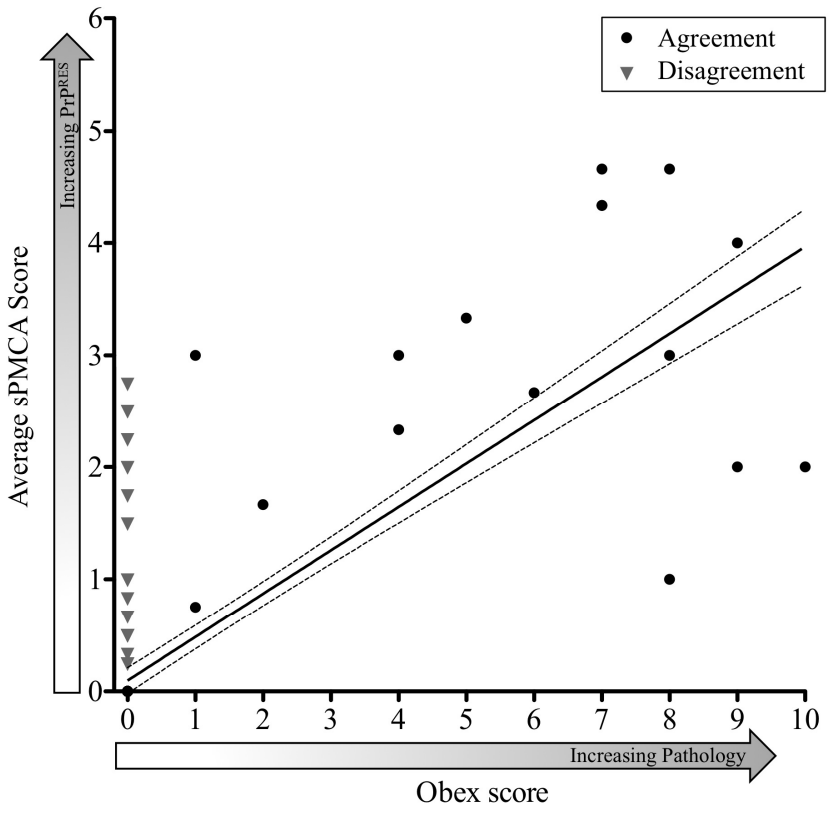

Figure 4.2. Correlation between obex score and sPMCA score of each sample.

The difference in sPMCA-positive results between 2010 (70.83%) and 2011 (18.18%) is believed to be attributable to cross-contamination between samples at time of collection in necropsy in 2010. The decontamination protocol applied in necropsy during 2011 to limit cross-

contamination reduced the sPMCA-positive rate to twice the IHC rate (sPMCA=18.18% and IHC = 9.09%, Table 4.1). The separate analysis of Trusted and Unknown samples allowed us to estimate specificity, or the probability of a true-negative given the animal is not sick, for those grouped Trusted and Unknown samples. Figure 4.3 shows the specificity of a negative sample giving a false-positive sPMCA result by round. If the sample is negative it will not be found positive in any of the 6 rounds, with a specificity of 94.17% (CI 90.07-96.97%) for Trusted samples and 64.13% (CI 53.03-76.99%) for Unknown samples. The specificity of sPMCA ideally would be low in each round, because a negative sample resulting in a positive in a round, such as the last round 6, would be a false-positive. The comparison of specificity for Trusted and Unknown samples in Figure 4.3 shows that as a sample is tested out to 6 rounds of sPMCA, the risk of a false-positive increases, particularly for our possibly contaminated, Unknown samples. Inversely, our specificity estimates show that sPMCA has a high specificity when cross-contamination is prevented. To estimate overall specificity of sPMCA, under the assumption that a sample is run 6 rounds, we again analyzed Trusted vs. Unknown samples and found similar results to values by sPMCA round (Figure 4.4).

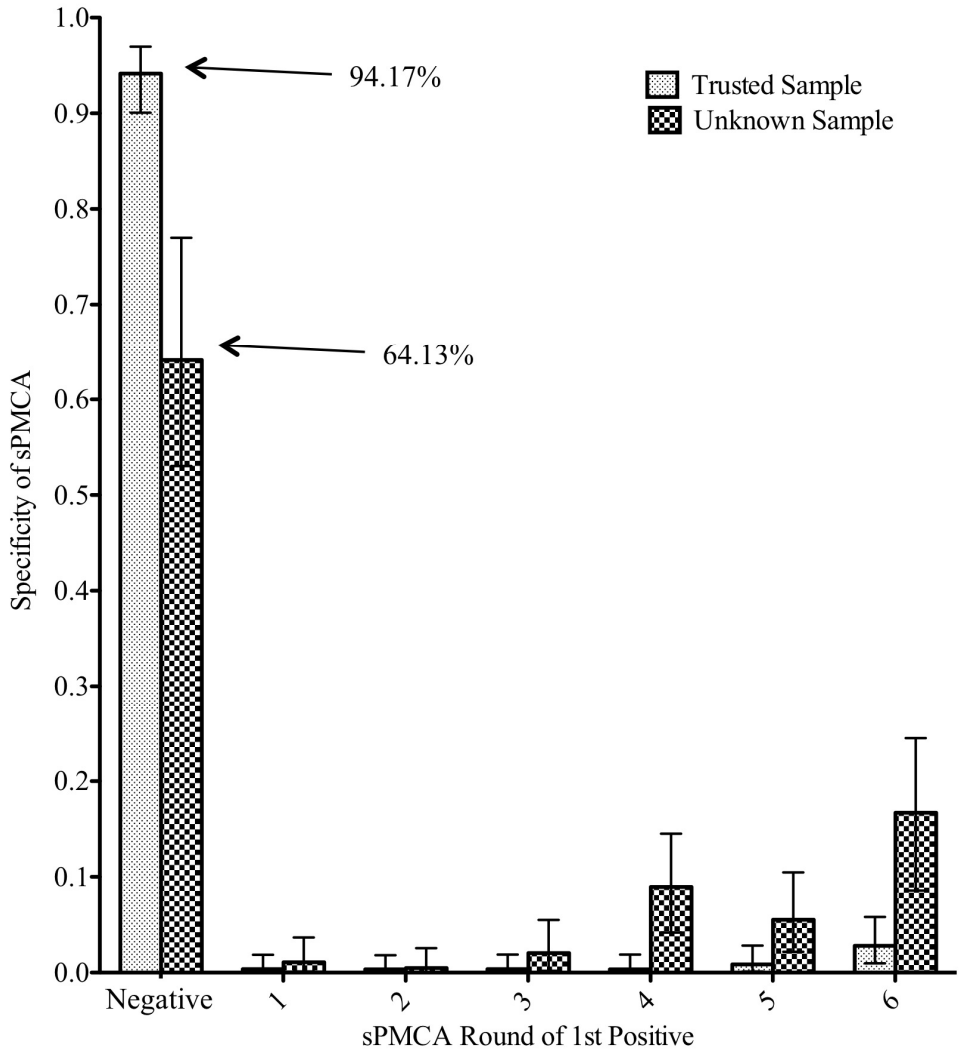

Figure 4.3. Specificity estimates for sPMCA by the first round that a sample would be positive, separated by Trusted and Unknown samples.

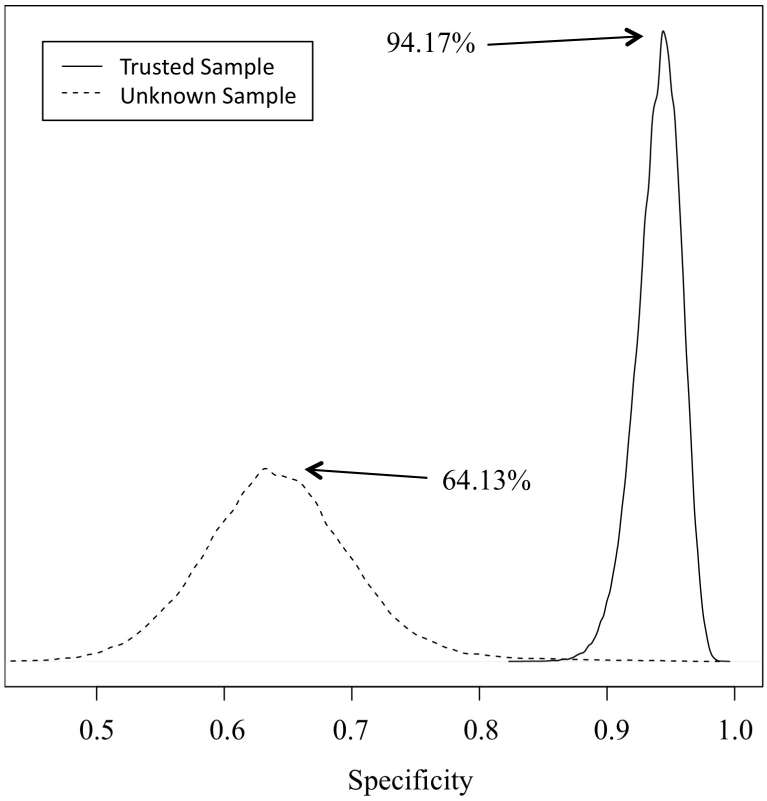

Figure 4.4. The overall specificity of sPMCA for Trusted samples is estimated at 94.17% (CI 90.08-96.96%) and for Unknown samples 64.13% (CI 53.03-76.99%).

Sensitivity estimates for IHC by tissue, obex, RPLN and SMLN are presented in Figure 4.5. When looked at separately, it appears that the RPLN (84.09%, CI 61.47-96.10%) is a more sensitive tissue for IHC sampling than obex (71.04%, CI 48.78-87.62%). The SMLN (7.10%, CI 1.05-21.92%) is estimated to have a very low sensitivity, because in this study only one of the 85 elk was positive by SMLN. Interestingly that animal was only IHC-positive in the SMLN. Though it did test positive by obex sPMCA.

The overall sensitivity estimate for IHC is the ability of all three tissues, obex, RPLN and SMLN, to be tested for one diagnosis (three-tissue IHC test). The sensitivity estimate for sPMCA assumes a sample is run for 6 rounds as outlined in our methods. We found that sPMCA on the

obex a nearly identical sensitivity at 95.83% (CI 82.92-99.79%) to the three-tissue IHC test 96.01% (CI 85.38-99.25%; Figure 4.5).

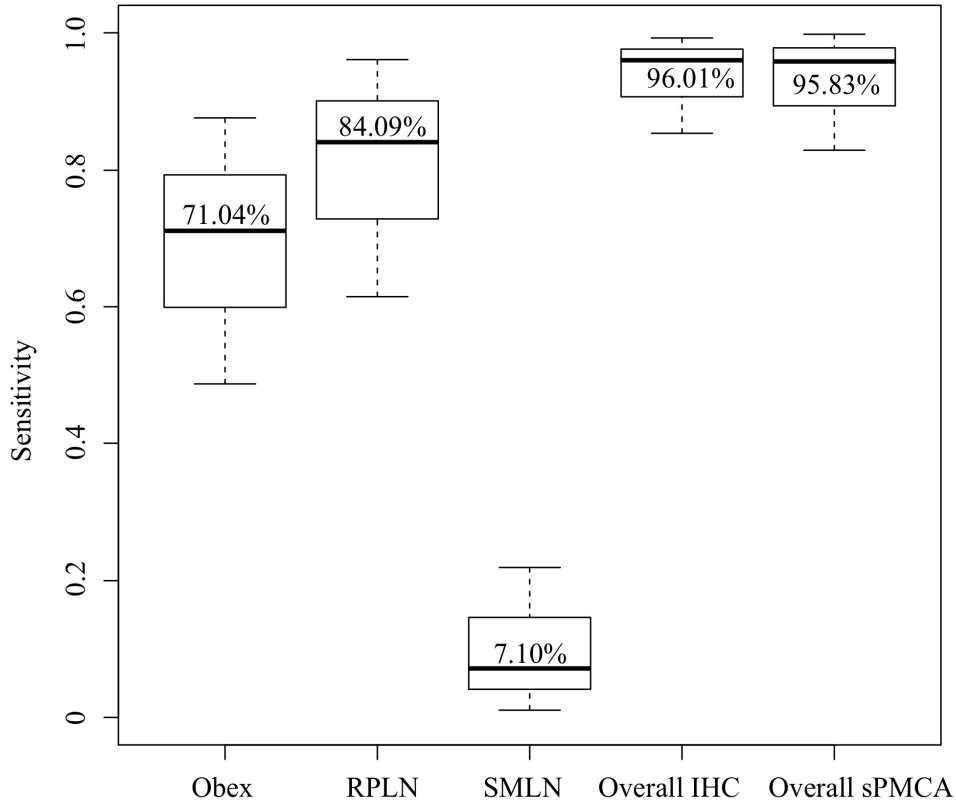

Figure 4.5. Sensitivity estimates for IHC by tissue (obex (71.04%, CI 48.78-87.62%), RPLN (84.09%, CI 61.47-96.10%) and SMLN (7.10%, CI 1.05-21.92%)) and overall sensitivity estimates for the three-tissue IHC test and sPMCA using 6 rounds.

The sensitivity of sPMCA by round is shown in Figure 4.6. We did not separate samples for sensitivity analysis because sensitivity is the ability of the test to correctly identify a true-positive as a positive. Sensitivity estimates imply that the ability of sPMCA to detect prions in a sample increases as more rounds are run. The greatest sensitivity was found for round 6, at 37.98% (CI 25.94-50.69%) indicating if a sample is a true-positive, it will be positive by round 6 if not before.

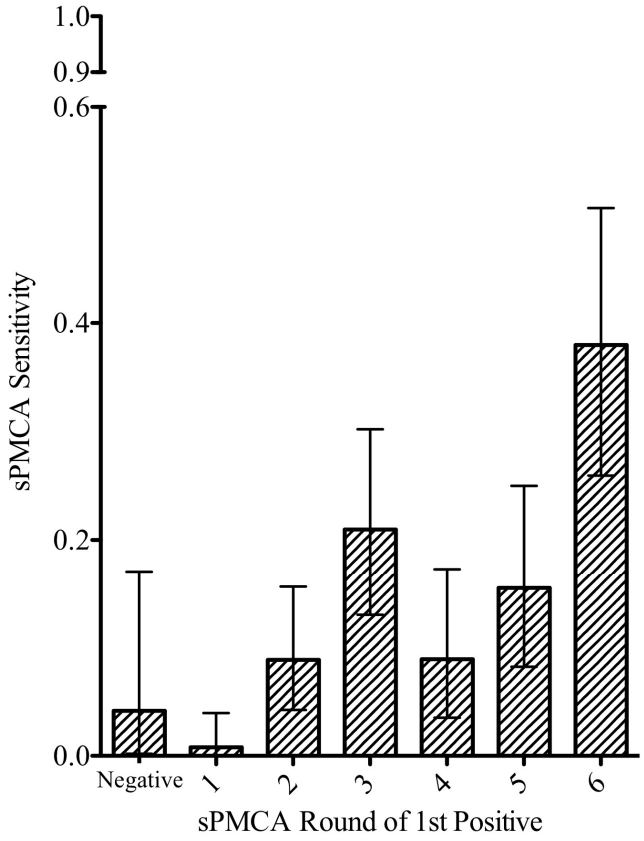

Figure 4.6. Sensitivity estimate of sPMCA by first round that a sample would be found positive.

To estimate CWD prevalence in the study population the model used all IHC results, and all sPMCA results adjusted for false-positives. With the information contained in both tests the prevalence for CWD in this population is estimated at 21.62% with a 95% CI of 12.23-34.54% (Figure 4.7).

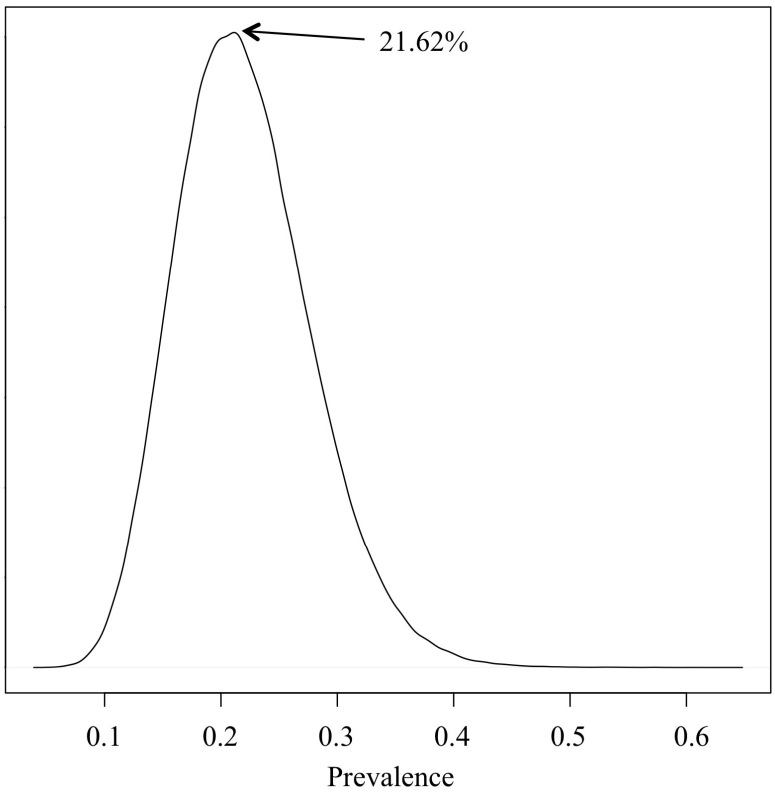

Figure 4.7. The CWD prevalence estimate of 21.62% (CI 12.23-34.54%).

The age distribution of elk sampled by year showed an overlap between years, with 2009 and 2010 including primarily middle-aged animals (Figure 4.8). The age effect on disease status in this study agrees with previous literature suggesting the primary age of infected animals lies between 2-11 years (Figure 4.9). Our data suggests the primary age cohort for infection would be juvenile to 10 years.

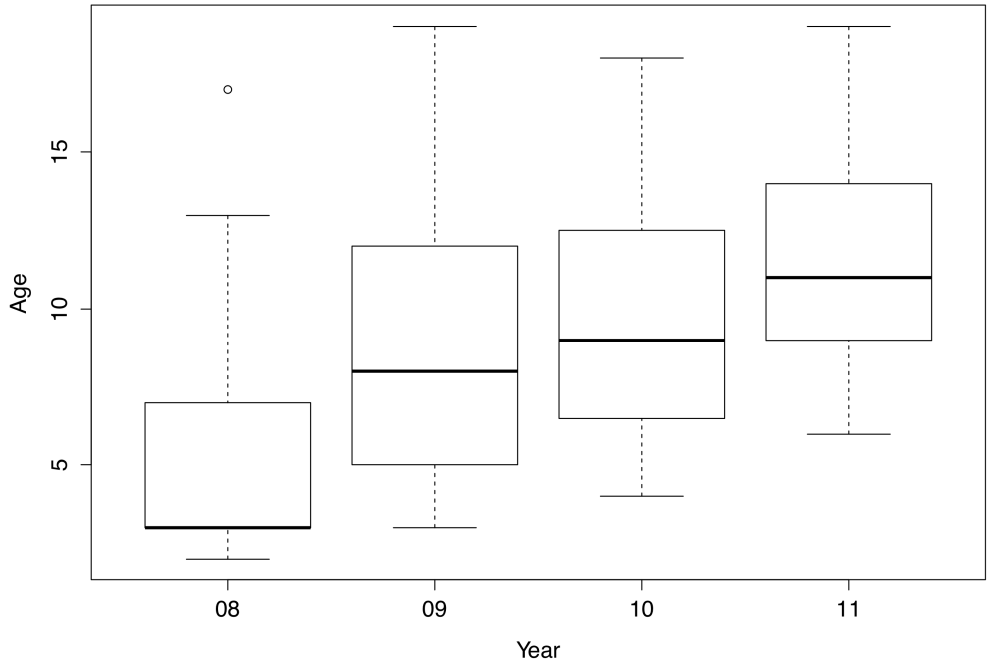

Figure 4.8. Age distribution of animals collected over the four years of the study.

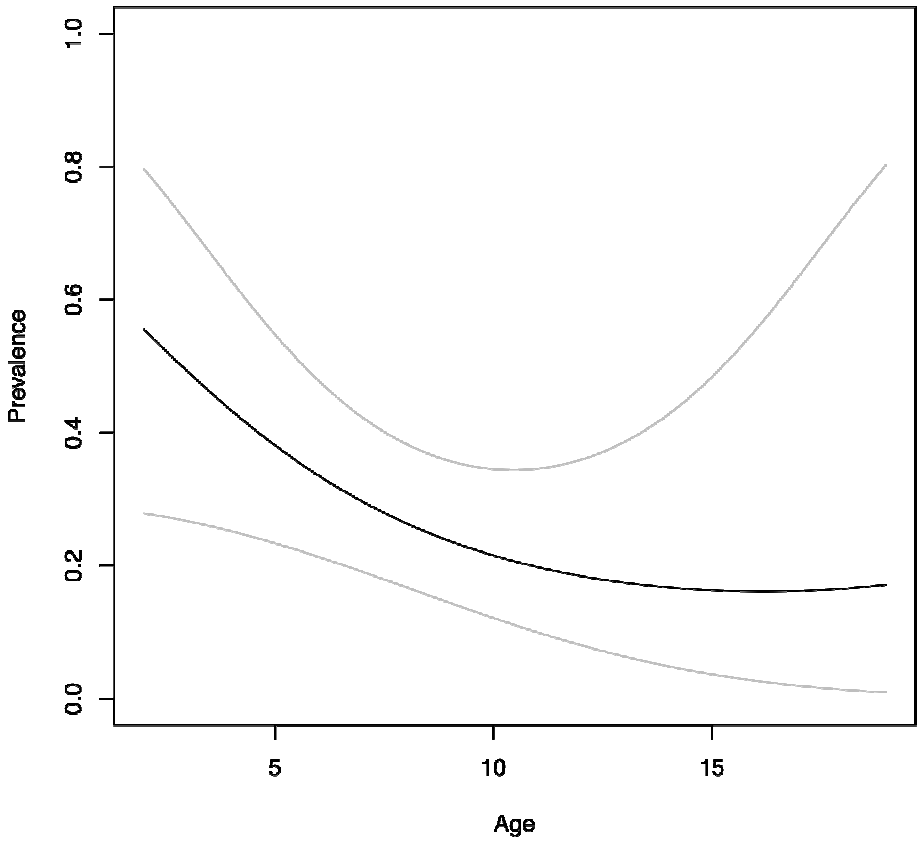

Figure 4.9. Age effect on prevalence, demonstrating the predominant age of infected animals lies between juvenile and 10 years as previously documented.

DISCUSSION

Our results suggest that CWD prevalence in free-ranging elk herds in RMNP is much higher than previously reported. Prior to 2013 the CWD prevalence in elk in and around RMNP was estimated at <3% [6,37]. In 2013 Monello et. al. [37] reported an IHC based CWD prevalence of 12.9% from these same samples, over 4 times higher than previous reports. Here we report an estimated CWD prevalence of 21.6% in this elk herd when modeling both IHC and sPMCA assay results. This nearly doubled prevalence suggests previous measurements have been missing a large portion of CWD-positive animals.

As an amplifying assay, sPMCA has previously been shown to be extremely specific and sensitive in other prion detection studies [23,44,45] but had not been directly compared to IHC in

elk or in samples from free-ranging animals. This study has shown that sPMCA on the obex alone is as sensitive at the three-tissue IHC test, and more sensitive than the IHC on any one tissue. In this study sPMCA also found several positive obex samples, which were IHC-negative from 2011. We argue that this increased detection represents early stage diseased or sub-clinical animals.

Similar to Monello et al. [37], our sensitivity analysis of IHC by tissue indicates that in this study population, that RPLN was actually more effective in detecting positives animals than the obex. These results indicate that the obex might not be the best tissue to test for CWD in elk, and perhaps the premise that the infection course is different between deer and elk is not entirely true. It is possible, that the testing of RPLN tissues by sPMCA would show a similar improvement on sensitivity compared to obex. Further studies are required to verify this trend in sensitivity.

Specificity of sPMCA in this study was lower than expected due to cross-contamination during necropsy. When we removed possible false-positive results for specificity estimates we found the value increased from 64.13% to 94.17% which is comparable to our previous findings of 99.59% in controlled laboratory experiments [35]. We believe that in the total absence of cross-contamination during necropsy, the model would have estimated a sPMCA specificity closer to our previous findings.

Concerns about cross-contamination during necropsy were addressed in the final year of sampling. The decontamination protocol mitigated the risk of cross-contamination for tissues assayed by sPMCA. This provided us with a year of samples, which we considered contamination free, and used to inform the model of sPMCA specificity and sensitivity compared to IHC. Unlike frequentist statistics, hierarchical Bayesian modeling allows us to work with

authentic but possibly imperfect data and let the model test parameters to find the best estimates. Having to discount some of the sPMCA findings for 2009 and 2010 was not ideal, but the model allowed us to adapt to the realities of research. The effective application of our decontamination protocol to mitigate cross-contamination has been instrumental for ongoing and future research projects involving sPMCA. This may be a useful protocol for other establishments concerned about cross-contamination in their own sample collection.

Our data demonstrates that previous IHC based studies are possibly missing early stage or sub-clinical cases in sampled populations. It is widely accepted that IHC is sensitive enough to detect pre-clinical cases, but we propose that sPMCA can detect cases even earlier. The detection of test-positive, but very early, or otherwise sub-clinical cases raises the question of biological relevance at the population level. We propose that this population is ecologically important to the disease transmission cycle. In previous work (NWRC study, Chapter 5) we found sPMCA had a detection limit of $1x10^9$ which is much more sensitive than the sensitivity of a mouse bioassay at $1x10^4$. This suggests that animals found positive by sPMCA have much lower levels of PrP^{CWD} than animals with clinical disease, but are indeed infected.

It remains unclear how early infected, or sub-clinical, animals begin shedding prions into the environment. Through the use of a mouse bioassay Tamguney et. al. [25] showed asymptomatic deer were capable of shedding infectious levels of CWD as early as 10 months prior to clinical disease. Bioassays, both in mice and deer, have limited sensitivity so shedding could be occurring much earlier than 10 months post-infection but at levels insufficient to cause clinical disease in the infected host.

It is commonly stated in the literature that CWD is an invariably fatal disease, however, it may only be more accurate to state that once animals begin to show clinical signs they are certain

to succumb to disease or other associated causes of death such as depredation [4,46]. Perhaps, instead, there are many carrier states within the population, which may or may not contribute to the transmission and deposition of prions in the population and the environment. Further research is require to address the role of a carrier state in the ecology of CWD transmission.

The application of sPMCA will be important to research and diagnostic investigation, as well as state and federal surveillance programs for CWD in both naïve and endemic host populations. Increased sensitivity, and the need for only obex tissue, might detect new focal points prior to clinical disease emerging in otherwise CWD free populations. Additionally, in the economically and politically difficult scenario of culling a captive herd that tested positive for CWD, extremely sensitive assays such as sPMCA are essential to verify that more than the index case were positive, and those sub-clinical carriers were likely shedding into the environment.

Overall, our data contributes to the increasing evidence that a portion of the population in an infected herd may become infected but due to age, genetic susceptibility or possibly some level of immune suppression, the animal remains sub-clinical and dies from other causes. However, the contribution of prions shed into the environment from this sub-clinical population may be important and requires further investigation. The existence of an infectious CWD carrier state aligns with disease ecology theory, which proposes there are trade-offs between transmissibility and virulence of a pathogen. As such, through selection pressures from the host and external environment the pathogen will tend towards the greatest transmissibility strategy. Despite the fact that prions are only protein, studies continue to point at evolutionary behavior and selection pressures of prions which indicate prions, like other pathogens, are capable of evolving and adapting to their environment [47]. With rapidly increasing prevalence at the

population level, as is reported in this study, sPMCA will continue to be an important tool to investigate CWD in wildlife.

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CHAPTER 5:

CHRONIC ENVIRONMENTAL EXPOSURE TO PRION CONTAMINATED SOILS RESULTS IN TRANSMISSION TO SUCEPTIBLE MICE

SUMMARY

Studies suggest that environmental deposits of chronic wasting disease (CWD) prions play an important role in the transmission and persistence of CWD among captive and wild cervids. Furthermore, studies indicate that the prion molecule forms a close association with clays and other soil components, enhancing its persistence and surprisingly, enhancing the transmissibility of the infectious agent. Investigation of PrP^{CWD} presence in soil has been particularly challenging due to limited sensitivity of existing laboratory assays. To date, detection of prions in field samples has been unsuccessful. Experimental studies of indirect transmission of CWD from environmental PrP^{CWD} contamination are required to understand the disease ecology, epidemiology and overall maintenance of the disease in wild populations. The goal of this study was to 1) investigate the role of indirect transmission by exposing PrP^{CWD} susceptible transgenic mice to PrP^{CWD} contaminated soil, 2) estimate average soil ingestion by laboratory mice, 3) estimate annual exposure to prions from chronic environmental exposure of prion contaminated soil and 4) evaluate the dose response of a single dose compared to chronic exposure. A prion-soil titration curve was created by orally inoculating mice with one of 5 dilutions of PrP^{CWD}-positive elk brain homogenate with 10% whole soil in sucrose. Second, additional groups of mice were either orally inoculated with, or housed on, soil originating from captive cervid research facilities where CWD occurred in herds. These soils, one from the Colorado Division of Wildlife research facility and the other from the Wyoming Fish and Game research facility, were considered “naturally contaminated” with PrP^{CWD}. Data from time point

sacrifices and clinically ill mice indicate mouse bioassay successfully demonstrated the presence of prions in naturally contaminated soil samples. We used bioassay findings to also estimate the amount of infectious PrP^{CWD} consumed by mice housed on soil, and to demonstrate dose response of chronic exposure verse a single oral dose. We demonstrate, for the first time, the successful use of transgenic mice for detecting prions in naturally contaminated soils and demonstrating a dose related response to prion-bound soils.

INTRODUCTION

Chronic Wasting Disease (CWD) was first seen in captive Colorado and Wyoming cervid populations in 1967. Twenty years later CWD was identified as a transmissible spongiform encephalopathy (TSE). Prions, the putative infectious agents of TSEs, are defined as proteinaceous infectious particles that lack instructional nucleic acids [1]. The specifics of how CWD prions cause CWD pathology are still unclear. What is known is the normal form of the endogenous mammalian prion protein (PrP^{C}) changes conformation resulting in an infectious conformer (PrP^{CWD}), which propagates more infectious molecules by inducing neighboring PrP^{C} molecules to change conformation from an α helical rich structure, to the predominantly β sheet form. Clinical signs of CWD include the classic TSE spongiform destruction of neural tissues, and prion amyloid deposits in brain tissue [2]. The degradation of the neural tissues results in behavioral changes and pathology including chronic weight loss, ataxia and eventual death [2].

Chronic Wasting Disease is now known to affect several cervid species including elk (*Cervis elaphus nelsoni*), mule deer (*Odocoileus hemionus)*, white-tailed deer (*O. virginianus*) and moose (*Alces alces spp.).* Other species have been shown to be susceptible but only by experimental inoculation, those include orally inoculated (p.o.) red deer (*Cervus elaphus elaphus*) [3], p.o. inoculation of reindeer (*Rangifer tarandus tarandus*), intracranial (i.c.)

inoculation and p.o. inoculation of squirrel monkey (*Saimiri sciureus)* [4], and i.c. inoculation of cattle (n=2 of 14) [5]. Current data suggests a robust species barrier exists in humans preventing transmission [6-10]. However, in the host species the disease has been demonstrated to be infectious and horizontally transmitted between animals [11], through excreta [12], and through indirect transmission from contaminated environments and fomites [13-15]. This is similar to scrapie, as prion disease of sheep. Both diseases appear to be lymphotropic, orally transmissible and environmentally stable [13,14].

The exact routes of host exposure to PrP^{CWD} in the environment have not been entirely elucidated, but the presence of PrP^{CWD} in the excreta and tissues of infected animals suggests continued deposition of the infectious agent into the environment while the animal is alive, and the deposition of the agent when the animal eventually succumbs to disease and decomposes [16-19]. It is suspected that incidental oral exposure to these environmental deposits leads to eventual transmission in susceptible species [13]. Indirect transmission becomes particularly relevant when considering the browsing and grazing habits, mineral lick use, territorial marking and social grooming behaviors of cervid species [20-22]. Geophagy, intentional and unintentional, is common among many animals [23-28]. It was estimated that mule deer can eat between 7 and 30 g of soil/day [26] creating a probable link in the indirect CWD transmission cycle.

The PrP^{CWD} molecule is highly stable *in vitro* and resistant to many standard decontaminant treatments. This impressive stability of CWD and scrapie (PrP^{Sc}) molecules has led to documented persistence, in and on, fomites and the environment [13-15,29,30]. Johnson et al. [31], using hamster adapted PrP^{Sc}, demonstrated the tight adsorption that occurs between the PrP^{Sc} molecule and different soil particles. It was also demonstrated that not only is the adsorbed

PrP^{Sc} still infectious, but the soil-bound PrP^{Sc} had increased infectivity when orally inoculated [32].

The quantity and persistence of PrP^{CWD} in the environment has been a particularly challenging aspect to study due to limited sensitivity of existing laboratory assays. Previous research by Miller et al. [13] was the first to experimentally demonstrate the indirect transmission of PrP^{CWD} from environmental contamination to previously unexposed mule deer in a period of less than one year. The results of this study directly supported the hypothesis that environmental contamination results in indirect transmission, but questions regarding chronic exposure, infectious dose and dose response of indirect transmission persist.

Experimental studies of indirect transmission of CWD from environmental PrP^{CWD} contamination are required to understand the disease ecology, epidemiology and overall maintenance of the disease in wild populations. The goal of this study is to 1) investigate indirect transmission by exposing PrP^{CWD} susceptible transgenic mice to PrP^{CWD} contaminated soil, 2) estimate average soil ingestion by laboratory mice, 3) estimate annual environmental exposure of prion contaminated soil and 4) evaluate the dose response of a single dose compared to chronic exposure. This study will begin to measure and further elucidate the relationship of PrP^{CWD} soil contamination, indirect transmission rates, and will demonstrate a bioassay model for further environmental contamination studies.

METHODS

Mice

Tg(CerPrP)5037 mice express 6-fold more elk PrP^{C} in the CNS and 0.5-fold less in the periphery compared to wild type expressing mouse PrP^{C} and are susceptible to PrP^{CWD} infection by multiple routes [18,33]. Mice were used in this experiment as opposed to the natural host due

to shorter incubation times, smaller space requirements, greater tolerance to handling [34], feasibly larger group sizes and no *a priori* exposure to potential PrP^{CWD} environmental contamination. Animal work was approved by Colorado State University IACUC Protocol ID: 09-1580A, Approval Date: January 14,2010 and the USDA/APHIS/WS-National Wildlife Research Center IACUC QA-1709.

Soil

CWD-positive soils were collected with permission from Colorado Parks and Wildlife wildlife research pens in Fort Collins, Co and Wyoming Game and Fish wildlife research pens in Sybille, WY. These soils were considered "naturally contaminated" as they were from pens with endemic CWD in captive mule deer and elk, respectively. These soils will be referred to as "MD pen soil" and "elk pen soil". Twelve 5-gallon buckets of soil were collected from the top 1-inch of pens grounds. Negative control soil was collected from two sites. The majority of soil used in this experiment was collected with permission from private property located in Southern Colorado game management unit 861 where CWD has not been detected in wild populations. The second negative soil was collected in the CWD-endemic area of the Front Range near Fort Collins, and was only used for a subset of p.o. inoculations. All soil bedding was autoclaved to knock down ambient microbes to prevent illness and intercurrent death in mice.

Soil classification analysis of whole soil was conducted by the Colorado State University Soil, Water and Plant Testing Laboratory (Fort Collins, Co). X-ray diffraction mineralogy analysis of whole soil was conducted by K-T GeoServices, Inc. (Gunnison, Co). Whole soil analysis included XRD weight percentage for bulk (whole rock) and clay fraction (,4 mm), pH, percent organic material, and soil texture classification of basic elements [35]. The following definitions were used for clay mineral classification: *Mixed-Layer Illite/Smectite* (M-L I/S) – A

clay mineral group containing interlayered or interstratified Illite and Smectite. Mixed layer type was identified by the minerals involved (Illite and Smectite), the type of order or stacking along the Z-axis (random or not ordered), and the proportions of the minerals involved (10% Illite and 90% Smectite). *Illite and Mica* – Common non-expanding minerals which are hydrated silicates containing potassium, silica and aluminum. *Kaolinite* and *Chlorite* – Common non-expanding hydrous aluminum silicate clay minerals.

Soil ingestion estimate

Mice were placed on negative soil for 48 hours. During the 48 hour period, mice were observed for 3 separate, 20-minute periods. After 48 hours mice were moved to a bedding free cage for 48 hours. Fecal matter was weighed to estimate average production per day and food was weighed before and after 48 hours to estimate average consumption. Samples of feces (before and after soil ingestion), soil and food were collected and analyzed by inductively coupled plasma resonance (ICP) by the Soil, Water and Plant Testing Laboratory, Colorado State University, Fort Collins, CO. Samples were analyzed for minerals including titanium, aluminum, and silica which have been used in previous soil ingestion studies [26]. We used two similar methods for estimating soil ingestion. The first is following the methods of Arthur et. al. using mineral content of food, feces and soil samples with the following calculations:

$$S_{ing}\ \text{mg/day} = \frac{((DW_{feces}\ \text{g/day})*(M_{feces}\ \mu\text{g/g}_{feces})) - ((DW_{food}\ \text{g/day})*(M_{food}\ \mu\text{g/g}_{food}))}{M_{soil}}$$

S – Soil
DW – dry weight
M – trace mineral estimate

The second method compared mineral content of feces from mice prior to being housed on soil to feces from mice after they were housed on soil for 48 hours. We again looked at minerals that would have limited host uptake for the estimation. We did not measure urine, so it is possible that these estimates are low due to unmeasured urine excretion of minerals from soil.

Chronic Exposure

Mice were housed on one of the three soil-bedding treatment types (negative soil, MD pen soil and elk pen soil) in 4-foot round stock tanks with approximately 1 cm of soil bedding for 1 year (Figure 5.1). Soil was exchanged every month for new pen soil, used pen soil was decontaminated by alkaline digestion at the Colorado State University Diagnostic Laboratory [36]. After one year mice were moved into traditional housing for an additional 235 days (600 dpi). Negative soil treatment included 10 mice, and each of the two naturally contaminated soils had 20 mice. All mice housed in stock tanks were female. Mice were given passive integrated transponder (PIT) tags to keep individuals identified throughout the study.

Figure 5.1. 20 mice housed in a stock tank on “naturally contaminated” soil bedding.

Single p.o. inoculation

The CWD field isolates used were E2 sourced from a clinical Colorado captive elk and D10 sourced from a clinical Colorado mule deer. One hemisphere of each brain was separately homogenized to a 10% dilution with sPMCA buffer (4 mM EDTA, 150 nM NaCl in PBS) in a commercial blender as previously described [37] and aliquots were stored at -80 °C until needed. E2 was the primary CWD isolate used in this study, D10 was used in previous studies.

Normal brain homogenate (NBH) was sourced from Tg(CerPrP)5037 mice and from a negative cow elk (eNBH) in Montana, a continuously negative population. Each were prepared as outlined in Meyerett et al. [38]. United States Veterinary Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors, USDA # 110166 Research.

Negative soil for p.o. inoculation was microwaved for 5 minutes and UV irradiated for 1 hour to kill environmental microbes. Inoculum was prepared at a 10% soil dilution in sucrose with dilutions of E2. 10% E2 homogenate was diluted into sucrose and soil to a resulting dilution of 1:50 (2%), 1:500 (0.2%), 1:2,000 (0.05%) and 1:10,000 (0.01%). Mixture was allowed to sit at room temperature for 24 hours to allow for prion adsorption to soil but avoid rotting of brain homogenate. Seven different p.o. inoculation treatments were used including 5 negative control mice p.o. inoculated with negative soil spiked with NBH, 4 groups of 45 mice inoculated with 1:50, 1:200, 1:2000, and 1:10,000 E2-soil, and two groups of 45 mice inoculated with either elk pen soil or MD pen soil. 12 hours before p.o. inoculation water was withheld from mice to increase oral ingestion of inoculum. Mice received 50 μl p.o. of E2-soil-sucrose inoculum by pipette to allow for maximum exposure to oral lymphatic tissues. Mice were kept separate for a few minutes after p.o. inoculation to allow for all inoculum to be ingested before joining cage

mates. P.o. treated mice were housed in standard housing with normal bedding for the duration of the study.

Negative soil inoculated mice (n=8) were euthanized at 600 days post inoculation (dpi). Time points were used for treatment groups with the sampling strategy outlined in Table 5.1. Mice were euthanized outside of the schedule if animals scored out on clinical signs including sudden weight loss, tail rigidity, ataxia, loss of extensor reflex, rough coat, kiphosis, akinesia and/or hyper activity, or unrelated pain or distress that would be categorized as a USDA pain category of E.

Table 5.1. Euthanasia attrition schedule for mice treated with a single p.o. treatment.

	Single P.O. Treatments												
dpi	**Negatives[a]**			**E2[b]**	**D10[c]**	**Soil spiked with E2 CWD dilutions**					**Naturally contaminated soil**		**Total mice remaining***
	NBH	**eNBH**	**NBH soil[d]**	**1:50**	**1:50**	**1:50[e]**	**1:50[f]**	**1:200**	**1:2,000**	**1:10,000**	**Elk pen**	**MD pen**	
0 d	16	5	8	12	17	15	45	45	45	45	45	45	596
50 d	16	5	8	12	17	15	35	35	35	35	35	35	283
200 d	16	5	8	12	17	15	20	20	20	20	20	20	193
600 d	0												0

[a] Negative brain homogenates
[b] CWD elk isolate E2
[c] CWD mule deer isolate D10
[d] NBH spiked into negative soil from Southern Colorado
[e] Pilot study using E2 spiked into soil from the Front Range
[f] E2 spiked into negative soil from Southern Colorado

LD_{50} titration

To give perspective on infectivity of different concentrations of our primary CWD isolate, E2, through the efficient i.c. route we inoculated a dilution series into Tg5037 mice to estimated the LD_{50} as done with previous prion isolates [39-41]. The dilution series consisted of the 10% homogenate E2 diluted into 10% NBH for the final dilution of 1:100, 1:1000, 1:10,000 and 1:100,000. Days post-inoculation to clinical disease (DPI) were used calculate log infectivity titers of each E2 dilution. A linear regression was run on DPIs plotted against log dilution factor to estimate slope as shown in Reed and Munch [41].

Detection assays

Mouse brains were collected and sectioned, the outer half of the left hemisphere was frozen at -80 C° and the remaining brain was fixed in 10% buffered formalin. Protein misfolding cyclic amplification (PMCA) was used to detect PrP^{CWD} in frozen tissues after homogenization. We used a modified sPMCA protocol [38] and tested each sample in quadruplicate for scoring purposes [19]. Briefly, test samples were diluted 1:2 with Tg(CerPrP)5037 NBH substrate in 0.2 ml PCR tubes. Samples were placed in a 37 C° water bath in a Misonix 4000 sonicator horn (Qsonica Inc., Farmingdale, NY). Samples were sonicated at approximately 120 watts for 40 sec every 30 min for 24 hr, constituting one round. For each subsequent round, 25 ml of each sample from the previous round was added to 25 ml of fresh NBH. Samples were run for 6 rounds then tested by western blot as outlined in Meyerett et al. [38]. The detection limit for this modified protocol was tested and compared to bioassay sensitivity estimates based on LD_{50} calculations outlined above.

Fixed tissues were assessed by histological techniques including H&E, GFAP and immunohistochemistry (IHC) analysis [33,38,42]. Mice found positive by either sPMCA or IHC were considered CWD-positive, regardless of clinical signs.

RESULTS

Soil analysis

Soil analysis of the three primary soils used in this study classified all three soils as Sandy Loam soils (SLS; Table 5.2). Clay content was comparable between soil types with overall clay content ranging from 11.5% to 19.8% weight. The smectite clays, which include Mte, were collectively measured and categorized as M-L I/S. The negative soil contained less smectite clay (19.1%) than the MD pen soil (32.3%) and the elk pen soil (27.4%). The primary difference in soil composition was the quartz contents, with the MD pen soil having the highest quartz content at 66.6% weight compared to 35.9% and 29.3%. Most significant difference between soils was the alkaline pH of the elk pen soil at 8.9 pH while the negative and MD soils were more neutral at 7.5 and 7.0 pH, respectively. Additionally, the electrical conductivity, EC, a general measure of salinity in the sample, was two and a half times higher in the MD pen soil than in other two soils.

Table 5.2. Soil Component and Mineralogy Analysis

Mineral	***Negative Soil***[a]	***MD Pen Soil***	***Elk Pen Soil***
Quartz	35.9	66.6	26.3
K-Feldspar	9.3	3.2	4.0
Plagioclase	38.3	5.7	49.5
Amphibole	1.3	1.0	1.6
Calcite	1.3	1.1	2.8
Pyrite	1.6	2.6	1.2
Hematite	0.8	0.0	0.0
R0 M-L I/S 90S[b]	2.2 (19.1)[c]	6.4 (32.3)	4.0 (27.4)
Illite & Mica[b]	7.7 (67.0)	11.2 (56.6)	8.5 (58.2)
Kaolinite[b]	1.4 (12.2)	1.6 (8.1)	1.4 (9.6)
Chlorite[b]	0.2 (1.7)	0.6 (3.0)	0.7 (4.8)
Total	100.0 (11.5)[d]	100 (19.8)	100 (14.6)
Soil Characteristics			
Texture class	Sandy Loam	Sandy Loam	Sandy Loam
% Sand	72.0	74.0	80.0
% Silt	14.0	10.0	8.0
% Clay	14.0	16.0	12.0
Ph	7.5	7.0	8.9
EC (mmhos/cm)[e]	4.6	3.5	11.6
% Organic Material	3.6	4.6	9.9

[a] % weight of whole SLS
[b] clay classification
[c] % of total clay weight
[d] clay weight % of total
[e] electrical conductivity (EC), measurement of salinity
[f] NA, not applicable

Soil ingestion estimates

Behavioral observations of mice housed on soil demonstrated soil ingestion during digging, sniffing and foraging, as well as self and mutual grooming. The introduction of soil as bedding initially provided novel environmental enrichment for mice, which lasted the entire period of observation, but did slightly decrease over the 48 hr experiment.

Soil ingestion estimates based on mineral measurements found in food, feces with soil and soil were comparable to estimates from comparing feces with and without soil. Food

ingestion was measured at approximately 3.5-5g/day/mouse and fecal production was estimated at 1.2-2 g/day/mouse. Soil ingestion estimates are outlined in Table 5.3. Comparison of ingestion ranges estimated with the different minerals and estimated methods showed variation, but contained a range of overlapping values with the exception of titanium (Figure 5.2). Using these estimates we approximated that mice are eating either 10-46 mg/soil/day according to estimates based only on titanium or as high as 70-1017 mg/soil/day based on multiple minerals. By these measures, a 25 g mouse would ingest between 0.004 and 4% of their weight in soil daily. If we take a conservative range based on overlapping estimates we estimate that mice were eating 46-191 mg/soil/day which is approximately 0.18-0.76% of their body weight.

Table 5.3. Mineral analysis results of soil, rodent food and mouse feces with estimations of soil ingestion at the low range (3.5 g/food/day and 1.2g/feces/day) and the high range (5 g/food/day and 2 g/feces/day).

	Mineral content μg/g				**Method 1 Ingestion Estimates mg/day**		**Method 2 Ingestion Estimates mg/day**	
Mineral	**Soil**	**Food**	**Feces**	**Feces w/soil**	**Low range**	**High range**		
Titanium	1141	5.8	0.001	26.20	10.27	20.51	27.55	45.92
Aluminum	14800	210	0.05	0.194	109.05	191.22	116.76	194.59
Barium	112	9.8	24.4	32.10	46.43	135.71	82.71	137.86
Iron	1.86	0.03	0.07	0.18	57.00	106.45	70.32	117.20
Manganese	464	78.9	0.02	236	32.20	167.03	610.29	1017.16

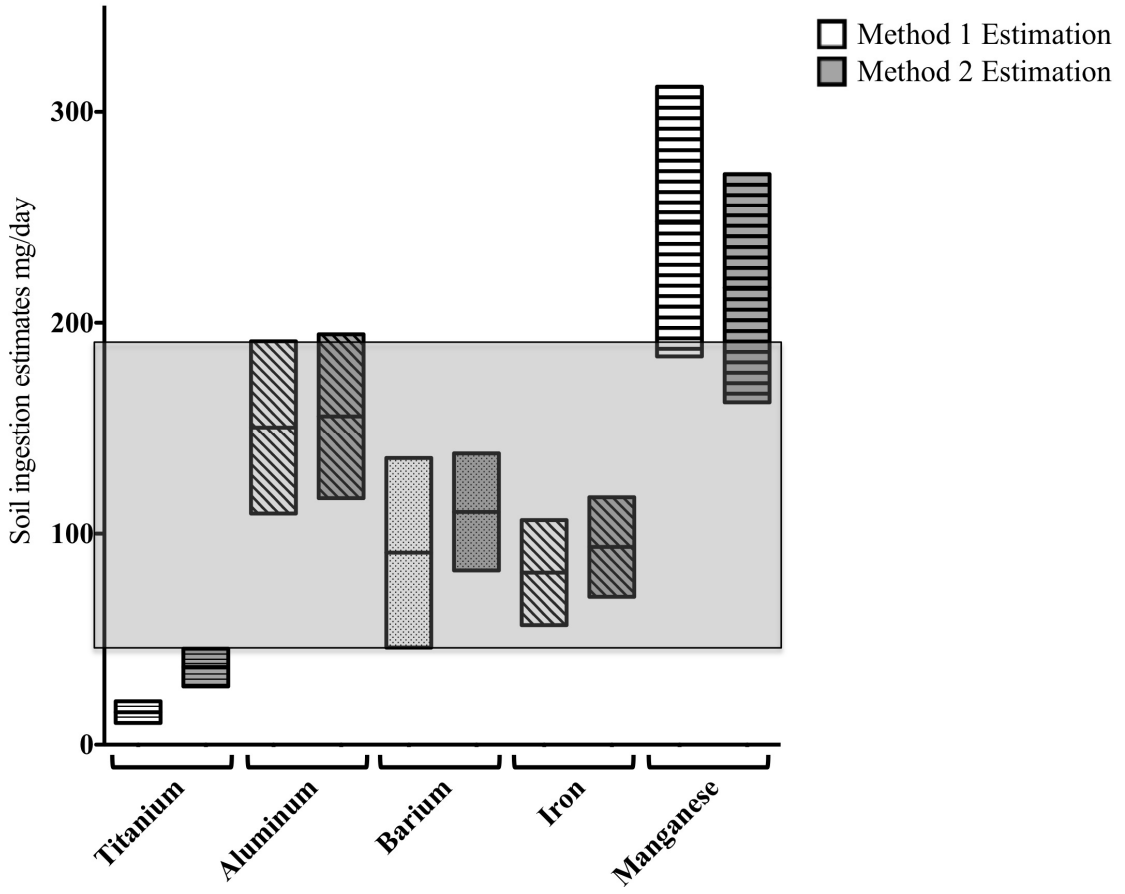

Figure 5.2. The range of estimated soil ingested according to mineral content analysis.

LD_{50} calculation

The LD_{50} i.c. titration was estimated based on clinical disease incidence in our four, dilution treatment groups. The $1x10^2$ and $1x10^3$ dilution caused clinical disease in >90% of mice, the $1x10^4$ dilution resulted in ~ 85% mice with clinical disease and ~ 50% at 1:100,000 (Figure 5.3). The infectivity titer of $1x10^5$ E2 is estimated at $4.6x10^4$ LD_{50} units of CWD prions with a linear regression equation of y=10.78-0.03x and an R^2 value of 0.5 (Figure 5.4).

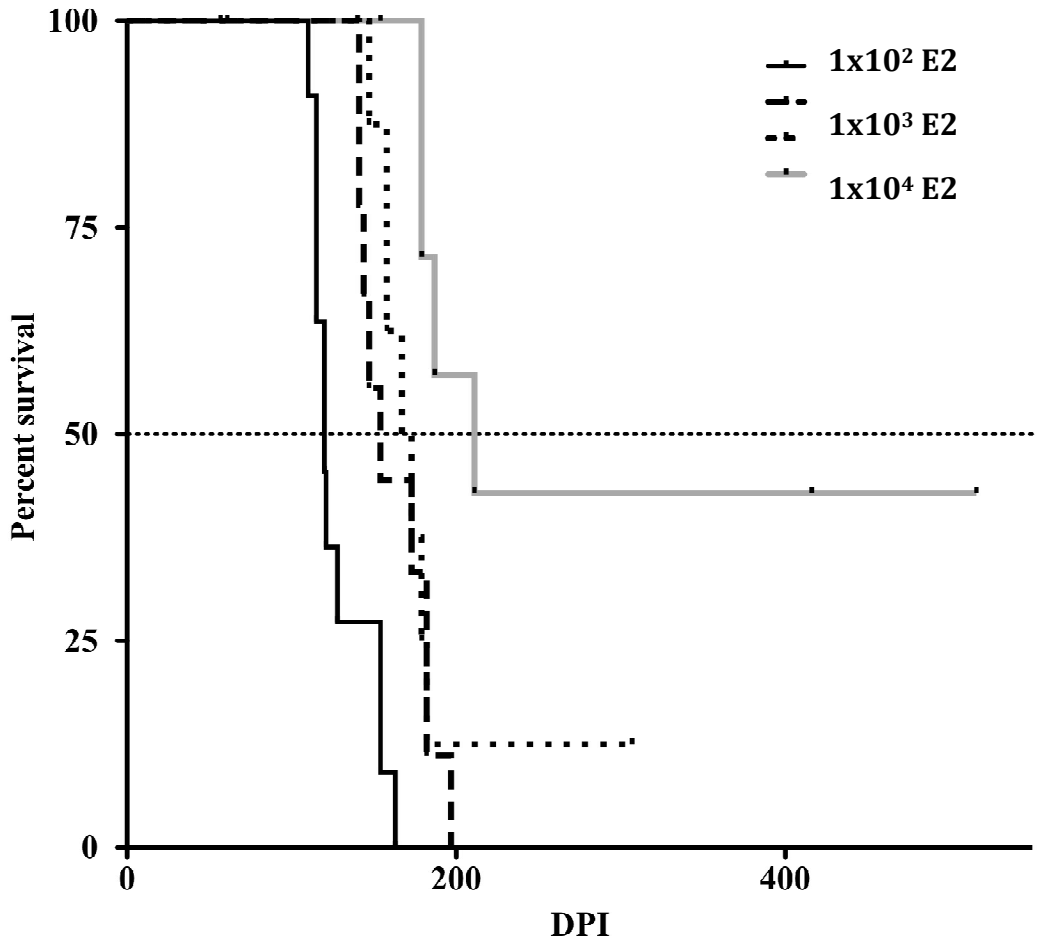

Figure 5.3. Kaplan Meyer survival curve for LD_{50} bioassay titration for E2 CWD isolate. 10% E2 was diluted into 10%NBH at the respective dilutions and i.c. inoculated into susceptible mice.

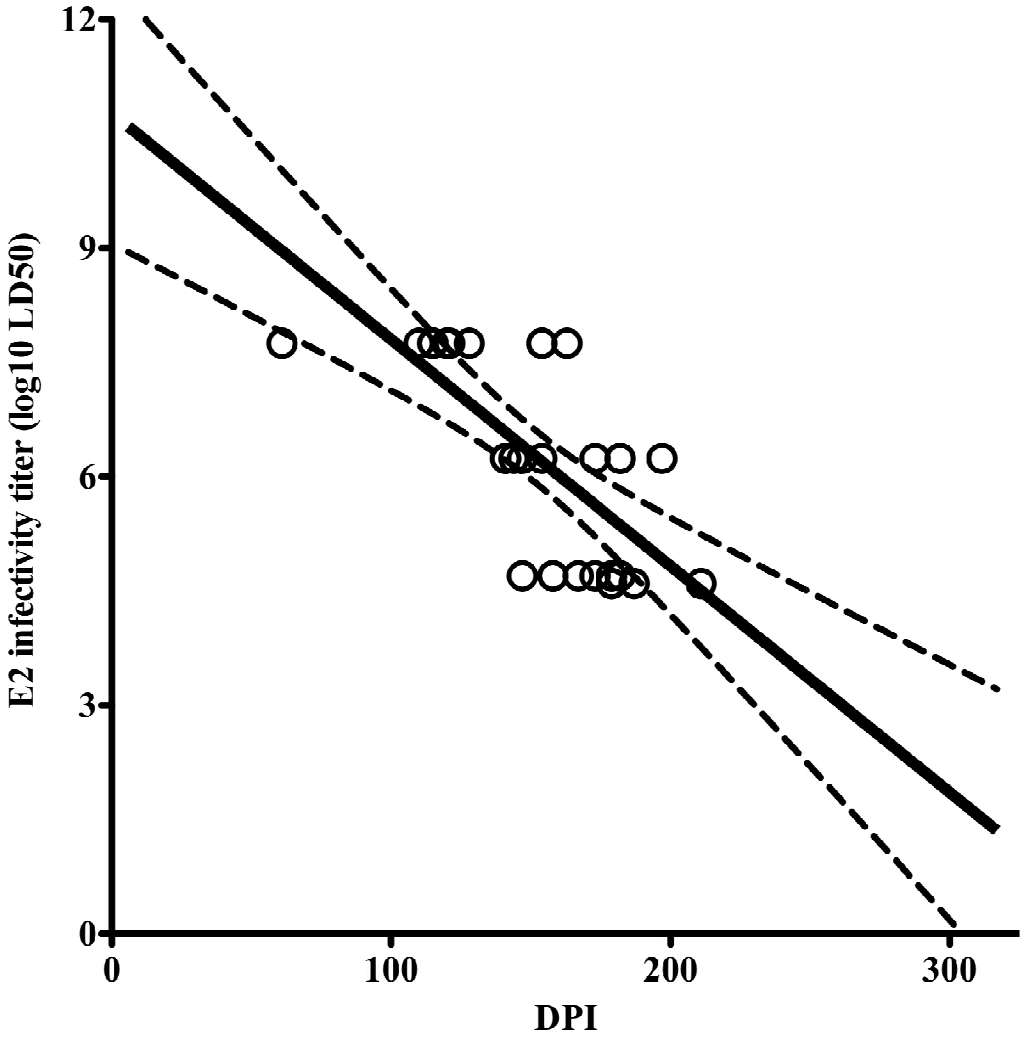

Figure 5.4. LD_{50} infectious titer as calculated from days post inoculation to clinical disease (DPI) [41].

Disease Incidence

Disease incidence and PrP^{CWD} detection are summarized in Table 5.4 (due to technical difficulties some sample results are pending for sPMCA). Clinically ill mice were rare, with only 6 mice showing sufficient clinical signs to be scored CWD-positive. Two of 60 mice demonstrated clinical illness in the 1:50, or 2%, p.o. treatment and one of 45 mice in the 1:500, or 0.05%, p.o. treatment group showed clinical signs. Each of these three animals received one single dose of E2 spiked soil. The remaining 3 mice that demonstrated clinical signs were from

the chronic exposure treatment, one housed on MD pen soil and the other housed on elk pen soil. Sufficient clinical signs were not observed in any other individuals or treatment groups to be considered CWD-infected. Infection status of clinically ill animals was verified by sPMCA.

Table 5.4. Disease incidence by clinical signs, sPMCA detection and IHC.

Single p.o. Treatment	Total # Mice w/Clinical Symptoms	Total # sPMCA +	% Positive by sPMCA
NBH[a]	0/16	ND	ND
eNBH	0/5	ND	ND
D10	0/17	ND	ND
D10p2	0/10	ND	ND
E2	0/12	0/1	0
NBH + soil	0/8	0/7	0
E2 + soil	1/45	2/29	6.9
+ FR soil	1/15	2/10	20
E2 (0.2%) + soil	1[c]/45	0/28	0
E2 (0.05%) + soil	0/45	5/40	12.5
E2 (0.01%) + soil	0/45	0/23	0
MD[b] pen soil	0/45	1/16	6.25
Elk pen soil	0/45	2/16	12.5
Chronic Exposure			
Negative soil	0/10	0/2	0
MD pen soil	1/20	1/1	100
Elk pen soil	2[c]/20	2/13	15.3

[a] Unless otherwise indicated all inoculums were a 2% (1:50) dilution of brain homogenate
[b] The concentration of prions is unknown in pen soils
[c] Infection status in clinical animals has not yet been confirmed by sPMCA in these animals

In vitro sPMCA detection sensitivity was reproducibly demonstrated out to $2.5x10^8$ E2 diluted into NBH, as compared to $1x10^5$ detection by *in vivo* clinical disease in the mouse bioassay. The *in vitro* detection sensitivity of brain samples from sub-clinical i.c. inoculated animals has not been quantified by sPMCA. The higher sensitivity of sPMCA resulted in higher incidence of CWD infection in all treatment groups as compared to clinical disease. sPMCA detected at least one positive in each treatment group, demonstrating low but successful

transmission of CWD prions through a single oral dose and chronic exposure. Overall, however, we found lower numbers of sPMCA positive samples than were expected due to a lower than expected transmission efficiency.

In this study IHC was of limited efficacy due to poor sample condition which resulted in limited to no antigen retrieval and staining. In the majority of tissues evaluated we encountered extensive spongiform-like pathology present in animals from this study as well as animals sampled from our prion free breeding colony (Figure 5.5). This spongiosis did not correlate with astrogliosis or PrP^{CWD} staining in samples of sufficient quality so we did not consider it a marker for CWD transmission. Additionally, background IHC staining was often found in the olfactory bulb of both control and treatment animals and is believed to be residual, undigested PrP^{C}. These findings were considered false positives and contributed to the difficulty in evaluating IHC staining of treatment animals.

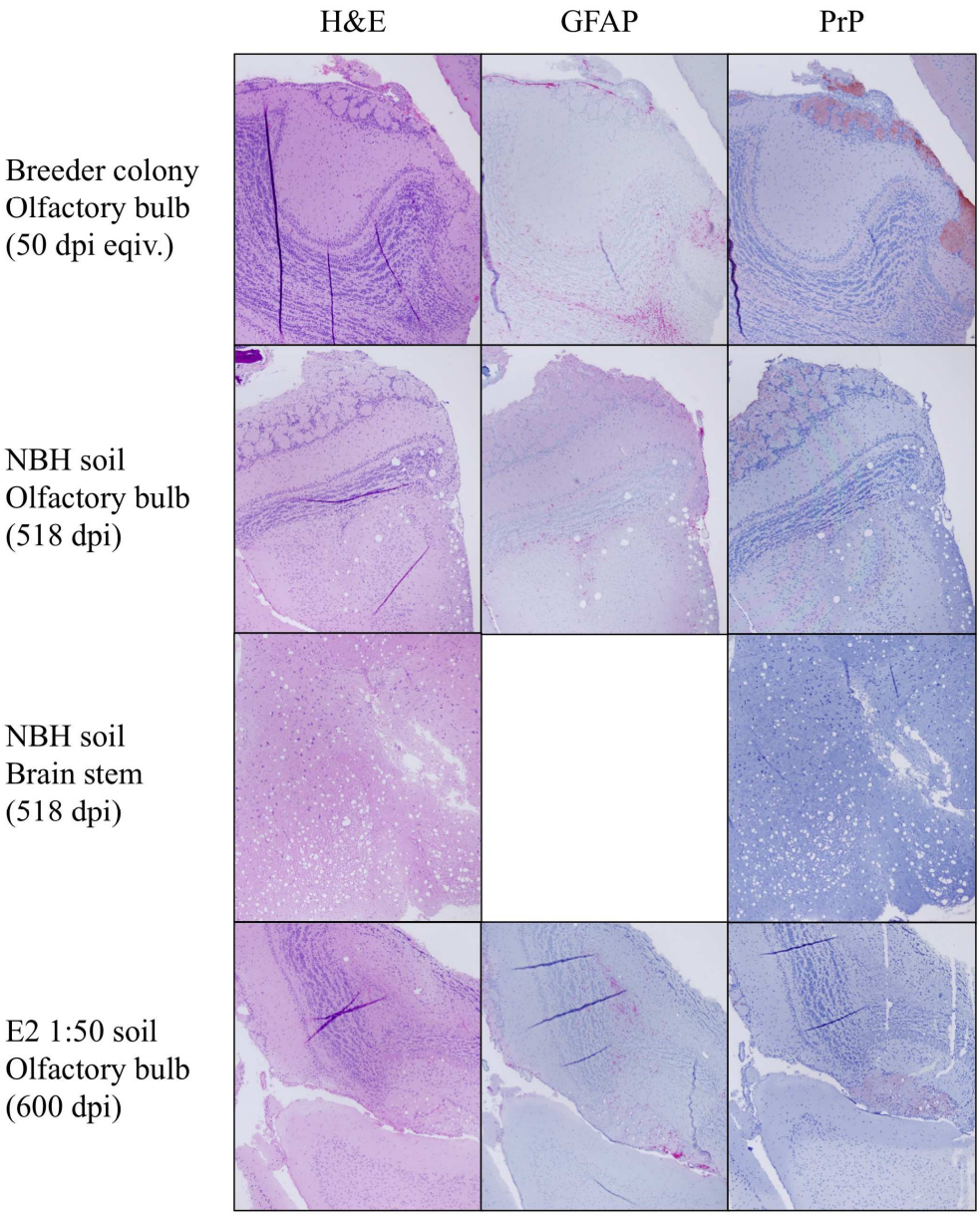

Figure 5.5. Representative histology images from prion free breeder colony, negative and positive soil treatments. Extensive spongiform-like pathology was present in many of the mice, and background staining in the olfactory bulb was commonly seen in negative controls and treatment mice, rendering histological diagnosis unreliable for this study.

DISCUSSION

Here we have demonstrated, for the first time, the use of a transgenic mouse bioassay to detect prions in naturally contaminated soil. We observed clinical disease and *in vitro* detection

of PrP^{CWD} in mouse brains, demonstrating limited, though infectious, transmission of CWD sourced from the natural host and the environment. This study also supports the debated hypothesis that CWD prions bound to soil are more infectious [43,44] than prions without soil. Of the 39 mice orally inoculated with one of two soil-free CWD isolates, none demonstrated clinical illness. However, mice inoculated with the same concentration of CWD prions plus soil resulted in 4 of 37 (10.81%) mice becoming clinical. The reason for this remains unclear but it is speculated that the soil could protect the prion from intestinal digestion, or it may enhance uptake by the immune surveillance cells of the gut associated lymphatic tissues [32].

Here we also report that chronic exposure to naturally contaminated soil is sufficient for CWD transmission in prion susceptible mice. This was previously shown in captive mule deer, a natural host species, however, this indirect form of transmission is a poorly quantified component of CWD transmission. We observed 6 clinical cases of CWD in treatment mice. Two individuals were inoculated with 2%, one with 0.2%, one was housed on MD pen soil and two were housed on elk pen soil. Clinical signs are not generally sufficient for diagnosis, so these mice were sPMCA tested to verify the presence of PrP^{CWD}. Due to a low overall incidence of transmission, it is unclear if a dose response exists between the mice who received a single oral dose of spiked or naturally contaminated soil compared to mice chronically exposed to naturally contaminated soil. However, the trend, and previous research, suggest a higher dose, eg. 2%, would have a higher incidence than a 0.01%, which is what we showed here. Interestingly, the two chronic exposure treatments did not appear to be equally infectious. This trend held true when samples were tested by sPMCA.

sPMCA has been shown to be far more sensitive at detecting PrP^{CWD} than a bioassay alone [35,45,46]. The detection limit of sPMCA for our CWD isolate E2 is $2.5x10^8$ compared to

$1x10^5$ by bioassay. We detected PrP^{CWD} by sPMCA in individuals of most treatment groups, but though the higher dosed animals had a higher incidence (Table 5.4). We did not expect many clinical cases, but did expect to see a higher rate of sPMCA positive samples than we found. Previous transgenic mouse CWD research has show oral transmission to be inefficient or entirely ineffective. Despite the findings that soil improves the transmission efficiency, our results also indicate a single dose of soil bound prions has low efficiency, while chronic exposure is slightly more infectious, but also inefficient.

The low observed incidence could be a result of different digestive physiology in mice verse cervids and sheep. Mice have simple stomachs while deer and sheep are both ruminants with a four chambered stomach. However, previous experimental studies using mice orally inoculated with scrapie have shown transmission to Peyer's patches as early as one week with a large dose [47]. We propose that it is not differences in digestive physiology, but instead may be a result of different peripheral PrP^C expression levels in the transgenic mouse which affect the pathogenesis. Due to the robust species barrier CWD studies have only been effective in transgenic mice. Further studies on oral exposure may require knock-in mice that have more natural expression levels of PrP^C.

Typical experimental challenge studies use a single dose of infectious material, but we agree with Williams et al. [48] that this is not representative of a natural course of infection and likely underestimates the timeframe of the average natural infection. Instead the exposure dose, the overall amount of prions the host is exposed to, directly influences the disease time course and possibly the intensity. Thus mice housed on contaminated soil were more likely to become infected, and to contract clinical disease, than mice treated with the same soil in a single p.o. dose.

The difference observed between the chronic exposure treatments of MD pen soil and the elk soil could be due to titer differences. The Wyoming elk pens routinely have new cases of CWD in captive elk, while the Colorado mule deer pens have been housing fewer deer and noticed a decline in new cases within the captive deer. The difference between incidences at facilities could be dependent on soil differences. While both pens contained sandy loam soil, the pH of the elk pen soil was significantly higher at 8.9pH compared to the deer pens at 7.0pH. The pH of soils is known to affect the binding capacity of infectious prions to oxide surfaces [49]. It is possible that in combination with other unidentified soil interactions, a higher pH may result in a higher overall negative charge of the protein and a shift away from the isoelectric point. This electrostatic repulsion could reduce the binding affinity of prions to soil components. It the lack of binding could result in increased degradation of free prions or alternatively if prions persist, it may change the infectivity of the prion upon ingestion. In light of the complexities of clay and mineral content and the role of ionic strength of the soil, it is clear that multiple forces are acting on the prion in the environment.

Alternatively, it could be due to CWD strain differences or host PrP sequence. The mice used in this study were transgenic for the elk Prnp gene, and incidence was highest in mice housed on the elk pen soil as compared to the MD pen soil. Strains in CWD are not as well characterized as those found with scrapie, however, there is evidence that variations exist within CWD isolates and that the cervid host may play a role in the strain selection [50]. This hypothesis requires further biochemical and bioassay study to characterize the CWD material collected from these mice.

Through foraging, grooming and coprophagy mice consumed soil, but only a fraction of their body weight (<0.76%) and less than 0.3% of their diet. Our findings are within range of

previous reports soil ingestion estimates by cotton rats (90 g average body weight) at 45 mg/soil/day [51] and for white footed mice at <2% of daily diet [28]. Our estimates suggest that over the course of a year mice could incidental ingestion of ≥ 3.65 g of soil.

Previous work by Wyckoff et al. [35] demonstrated the binding capacity of RML, a mouse-adapted scrapie strain, to a sandy loam soil when incubated in excess for 24 hours was substantial and likely not saturated [35,52]. Through a subtractive assay using intracranial inoculation of susceptible we conservatively estimated that the soil could bind ~$9.8x10^5$ LD_{50} units RML infectivity/g soil. If we assume a high level of contamination sufficient to infect 95% of elk in the Sybille pens, we could extrapolate that mice consuming a minimum of 10 mg soil/day of that contaminated soil would be exposed to 9,850 LD_{50}/day. Over the course of a year the mouse could consume up to $3.6x10^6$ LD_{50} units. At the middle of our estimate, 100 mg soil/day mice would have consumed 98,500 LD_{50}/day and over the course of a year the mouse could consume $3.5x10^7$ LD_{50} units. Due to the inherent difficulty of measuring prions in soil, we can only extrapolate using existing data. In the natural host, oral transmission from the environment and fomites appears to be efficient, so it is likely that the dose for infection could be considerably lower yet significant in transmission.

The quantity and persistence of PrP^{CWD} in the environment has been a particularly challenging aspect to study due to limited sensitivity of existing laboratory assays. Long-term environmental reservoirs of pathogens contribute to the epidemiology of many diseases including avian influenza [53], anthrax, hanta virus [54] and botulism. Evidence such as increased infectivity of soil bound prions [43], correlations of soil clay content with disease prevalence [55], and ever increasing disease prevalence within populations, suggests this is also the case for CWD. Our research findings are a significant step forward in assessing

environmental prion exposure and transmission. Without methods to measure environmental contamination, degradation rates, and rates of transmission over time it is difficult to forecast or estimate the ecology of the disease at the population and landscape level. We have shown for the first time mice can be successfully used to demonstrate prion infectivity of naturally contaminated soil. Further experiments, and new lines of transgenic mice will likely improve the efficacy of this bioassay technique. We have also shown that studies using single oral inoculation for infectivity studies may be underestimating the infectivity disease course of a given prion strain or preparation. Instead, multiple inoculations or chronic exposure should be used to model disease transmission ecology and more accurately represent what occurs in nature.

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OVERALL CONCLUSION

CWD is the only known prion disease of free-ranging wildlife. Since it's discovery in a captive herd over 40 years ago it has spread to 22 states and Canada and South Korea. It is believed that environmental deposition, and persistence of CWD prions play an important part in the disease transmission ecology and the increasing prevalence within populations. Only recently has the cycle of host prion shedding, environmental contamination, and indirect transmission become clear. As the evidence increases regarding the importance of this host-environment-host cycle plays many questions remain regarding this poorly understood disease ecology.

In the first part of this dissertation we investigated the binding capacity of RML prions to sandy loam soil (SLS) and pure montmorillonite clay (Mte). Through a subtractive infectivity bioassay we showed that SLS was capable of binding 95.3% of infectious prions while Mte bound 99.98% of infectious prions. This was the first time the binding capacity of the infectious prions has been measured, as opposed to the soil binding capacity of all components of prion homogenate, which may or may not be disease relevant. We believe this is significant because little is know regarding the changes in protein structure or infectivity that may occur during adsorption to soil. Additionally, this study adds to the evidence that Mte is an important soil component for binding prions and provides a method for further testing of individual soil components.

The difficulty of detecting prions in soil at natural levels has hindered progress in CWD research. At present detection of prions bound to soil has only been successful at high experimental doses with little relevance to the low levels expected to occur in natural settings. In the second part of my research we started the development of a new detection assay utilizing aptamers, small oligonucleotides, to directly bind PrP^{RES}. We applied aptamers to two different

detection strategies. The first was a direct visualization method, akin to immunodetection, using electrophoresis and western blotting. The second was as rtPCR amplification of aptamers that bound prions. Through the direct detection, the aptamers successfully recognized two of six prion strains tested. One of the strains aptamers did not recognize was E2, the CWD isolate used to select the aptamers. Despite this initial success with direct detection, follow-up experiments of successful results were inconclusive. Results from the rtPCR detection of aptamers was also difficult to repeat and often inconclusive. Additionally, our data suggests that the aptamers we developed had a non-specific affinity to an elk brain homogenate component, most likely DNA, which caused false-positives. We believe that with a more robust negative selection step at the time of aptamer design we might be able to remove this affect. Overall, though my early assay development was only partially successful, I believe aptamers still have the potential to be a sensitive and specific means of detection.

Studies suggest shedding of prions into the environment through host excrement contributes to an environmental reservoir of prions and indirect disease transmission. Currently, the disease burden of CWD-infected populations is estimated by IHC, a very specific detection test requiring specialized training and fresh tissues. In the third component of this dissertation I present data showing serial protein misfolding cyclic amplification (sPMCA) on elk obex tissue is as sensitive as a three-tissue IHC test and more sensitive than IHC on obex alone. Through my testing of samples from a free-ranging elk herd we found the IHC may be missing early, or sub-clinical, cases of CWD. We propose that these cases are ecologically relevant and have not been well characterized. We also show, through hierarchical Bayesian modeling, that prevalence estimates based on IHC results alone significantly underestimate the population prevalence of CWD, discounting the overall contribution of shed prions from sub-clinical animals.

Lastly, in the absence of a detection assay for prions in the environment we do not have the capacity to measure presence-absence or estimate the amount of prion contamination that exists in an area. There is also a debate regarding the oral infectivity of prions when bound to soil. To date, there is evidence for and against an increased infectivity of soil-bound prions compared to soil free prions. In the final section of this dissertation, I used mouse bioassay to test the oral infectivity of CWD prions with and without soil, at different spiked dilutions and at natural levels from naturally contaminated soils. We also tested the dose response of a single treatment compared to chronic exposure to contaminated soils. We found that soil-free CWD prions were not infectious while the same inoculum combined with soil caused clinical disease. Incidence data from different dose treatments were limited, as oral inoculation of CWD in mice appears to be inefficient, however, the two highest doses had incidence of clinical disease. Detection of sub-clinical disease in mice was also found by sPMCA in nearly every soil treatment group. The dose response effect of a single treatment compared to chronic exposure suggests chronic exposure is more efficient for disease transmission, which is more representative of natural transmission. The results of this study directly supported the hypothesis that environmental contamination results in indirect transmission, but questions of exposure rate, infectious dose and dose response of indirect transmission persist.

FUTURE DIRECTIONS

The success of the projects reported above has stimulated further questions. For the soil binding BASICS project, the use of a laboratory scrapie stain RML, was the first step in understanding the binding capacity of soil. Today, we are replicating the study using a LD_{50} titered CWD field strain, E2. We believe that use of a field isolate, particularly a CWD isolate will better model the natural system and be more relevant to future CWD research. Previous laboratory studies have shown that scrapie and CWD have different behaviors and persistence in soil, so it is important to also evaluate CWD by BASICS. Additionally, we were curious how different types of soil would affecting binding capacity and if the augmentation of those soils with Mte would increase the binding capacity. This study is ongoing, but preliminary results suggest that similar to RML, CWD binds to soil effectively removing a portion of the infectious doses from solution (Appendix A, Figure A1.1 and A1.2). The data also suggests that while Mte does play a role in binding, it might not be the only important soil component (Figure A1.3 and A1.4). Further investigation is required to estimate the relative contribution of Mte and other soil components to the binding of CWD.

Our findings in chapter 5, that soil bound prions are more infectious raises multiple questions regarding oral prion infection. The primary question is why would a prion bound to soil be more infectious than an unbound prion? It has been proposed that the action of binding soil may protect the prion from digestive processes, or that the prions that bind the soil may be optimal for infection through size or increased chance of uptake by immune surveillance cells. To test this difference in infectivity we have begun a study that utilizes enriched prion rods (Appendix B, Figure A2.1), which have been fluorescently tagged. Mice were inoculated with these fluorescent rods or with rods incubated with Mte to assess how Mte affected the early

stages of infection. These fluorescent rods allow us to image orally inoculated prion rods as they moved through live mice in real-time (Figure A2.2). Next upon necropsy, we were able to visualize the fluorescence was contained in the gastric tract (Figure A2.3). Unfortunately, the signal faded in live mice after 24 hours requiring us to utilize flow cytometry to assess the frequency of individual cells picking up the rods and moving them around. Preliminary tests using intraperitoneal inoculation showed that our inocula was picked up by cell populations and that the mock rods, made from normal brain homogenate, did not contain material for cell trafficking (Figure A2.4). Further studies taking cells from the digestive track after 24 and 48 hours post oral inoculation began to show prions were accumulating and being picked up by cells in the Peyer's Patches and in the small intestine (Figure A2.5 and A2.6). Further investigation using cell surface markers to individually identify cells trafficking prion rods indicated that at 48 hours a identifiable population of CD24+ intestinal epithelial cells were picking up the prion rods. Interestingly, this trend was primarily in animals inoculated with rods and Mte, and less so in rods alone inoculated animals. This is a significant finding because limited evidence exists implicating the intestinal epithelium. Even more significant is our discovery of a difference in trafficking patterns dependent on the presence of Mte. Further research is required to verify the cell type, test other time points and better understand why the presence of Mte affects prion uptake in the gut.

Overall, the host-environment-host prion cycle is fascinating and many questions remain regarding the increased infectivity of soil bound prions, the unknown timeframe of sub-clinical disease, the rate and length of prion shedding, as well as the possibility of a carrier state. Each question we answer leads to more questions as is typically the case. But as we compile evidence to explain how a protein can be infectious and how a genome-free pathogen can demonstrate

evolutionary behaviors such as selection and transmission strategies, we are challenged to shift our definition of a pathogen. Prions may have broken the molecular dogma, but in that effect they have expanded the infectious disease paradigm.

APPENDIX A.

CWD ADSORPTION TO DIFFERENT SOILS AS ESTIMATED BY BASICS

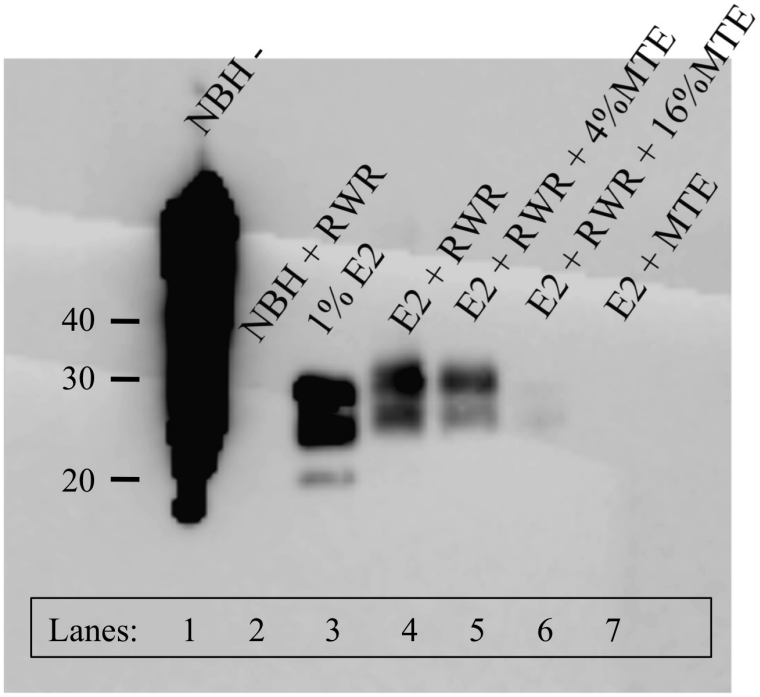


Figure A1.1. Western blot analysis of CWD-E2 inoculums treated with the BASICS protocol. In brief, inocula were incubated with different soil/clay treatments. Prions bound to soil were removed from inoculum by centrifugation and removal of soil pellet. Starting concentration of CWD prions in inocula is represented in lane 3, each inocula following started with the same amount of CWD, but treatment and removal of prions by soil reduced prion titer. Western blot analysis shows detectable levels of prions in all but 100% MTE treatments and a considerable decrease of prions in the RWR 16% Mte treatment. We also noted a difference in the molecular size of E2 (lane 3) and the prions that remain in solution after incubation with whole soil (RWR) a sandy loam soil (lanes 4 and 5).

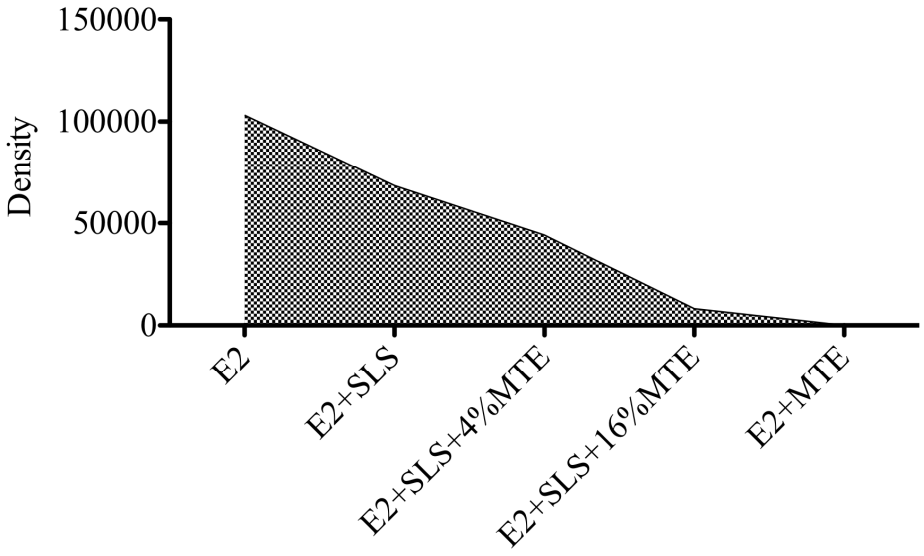


Figure A1.2. Densitometry analysis of the western blot image in Figure 1 indicates a decreasing signal in treated samples congruent with increasing augmentation of whole soil by Mte clay.

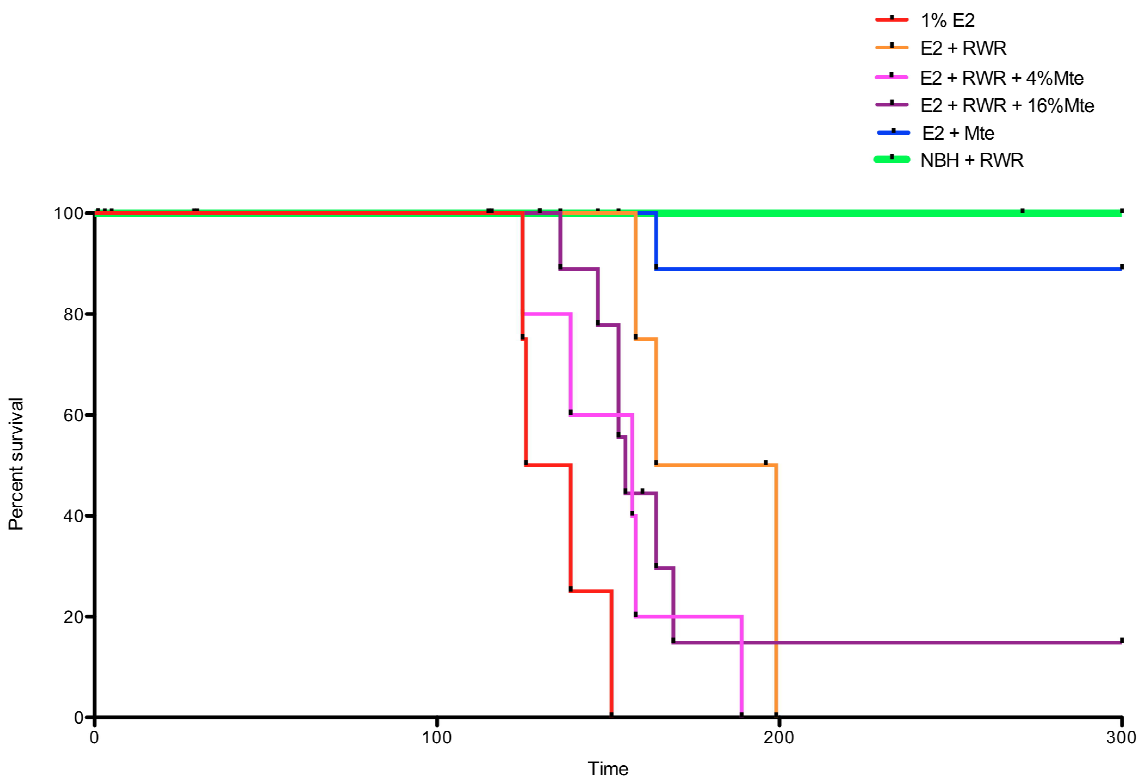


Figure A1.3. Kaplan-Meyer survival curve of mice inoculated with different E2 BASICS treatments. Survival curves demonstrate the delayed disease onset in mice inoculated with E2 that was soil treated. It appears that the addition of Mte to SLS soil does not increase the binding capacity. This study is still underway with animals surviving in the NBH+RWR and E2+Mte groups.

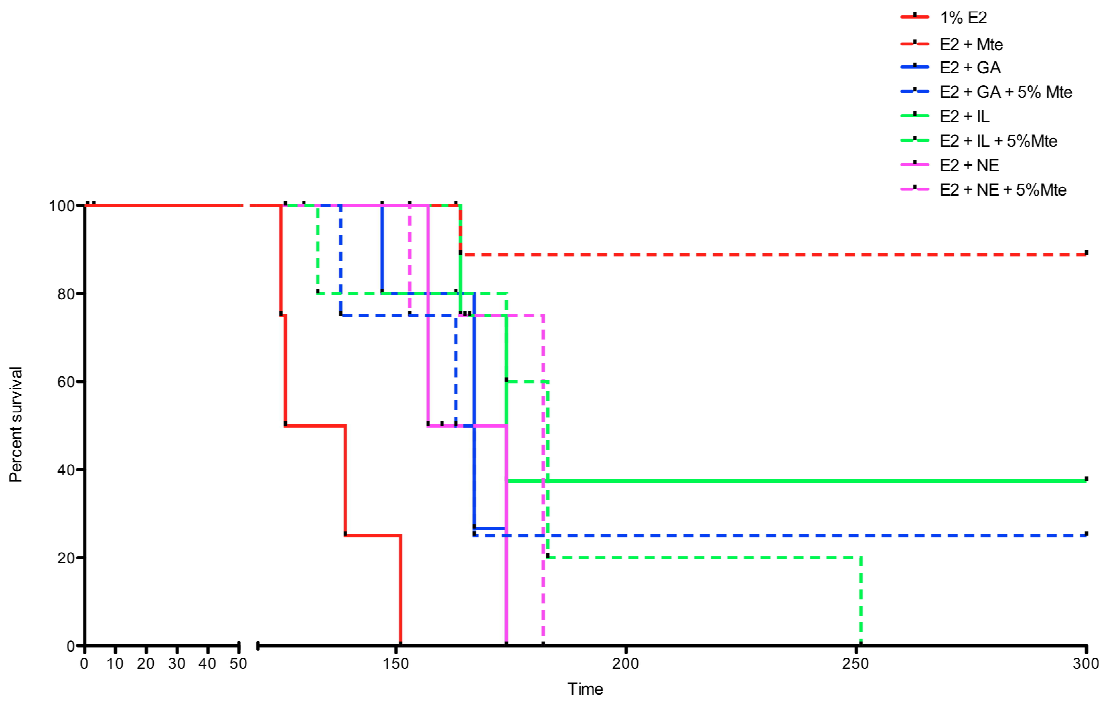


Figure A1.4. Kaplan-Meyer survival curve of mice inoculated with soil from Georgia (GA), Illinois (IL) and Nebraska (NE). Each of these soil types were incubated with E2. A replicate of each soil was also augmented with 5% w/v Mte and then incubated with E2 to see the effect of additional clay on binding capacity. Preliminary results suggest the addition of Mte may have increased the binding capacity of soil slightly. The study is on going with mice surviving in three treatment groups.

APPENDIX B.

THE ROLE OF MONTMORILLONITE CLAY IN PRION TRAFFICKING FROM THE GUT

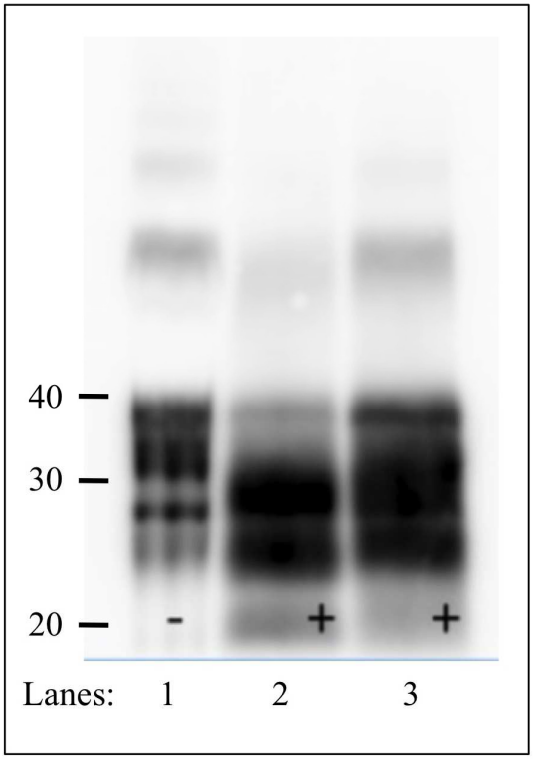


Figure A2.1. CWD-E2 isolate after enrichment protocol shows strong prion rods signal by western blot. Lane 1 – E2 rods no PK; lane 2 and 3 – E2 rods with PK.

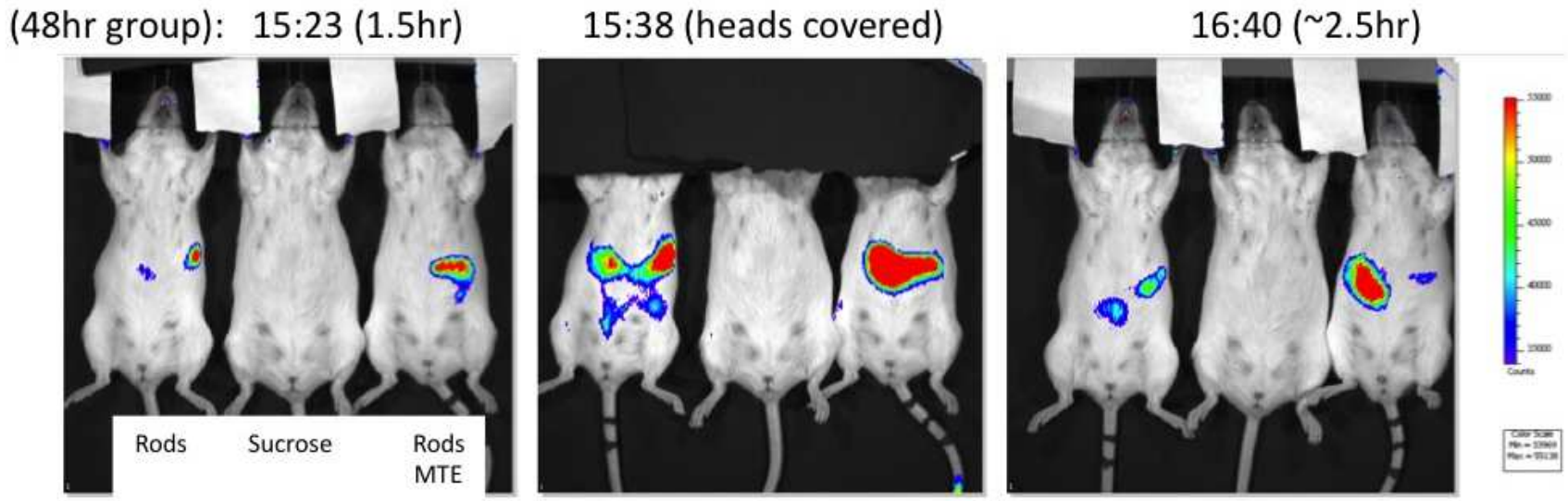


Figure A2.2. Live imaging of mice orally inoculated with fluorescently tagged E2 prion rods. The mouse on the left was treated with 50 µl of 1:200 fluorescent rods in sucrose, the mouse on the right received 50 µl of 1:200 fluorescent rods + 5% Mte in sucrose, and the mouse in the center only received sucrose. Fluorescent signal can be seen moving from the oral cavity, to the gut

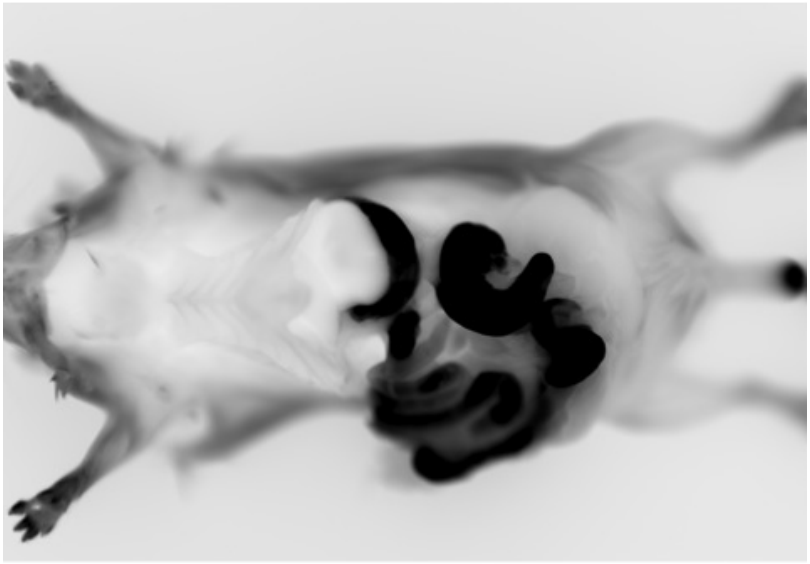


Figure A2.3. Upon necropsy, fluorescent signal (dark contrast) appears to be localized to gastric track, primarily stomach, small intestine and cecum.

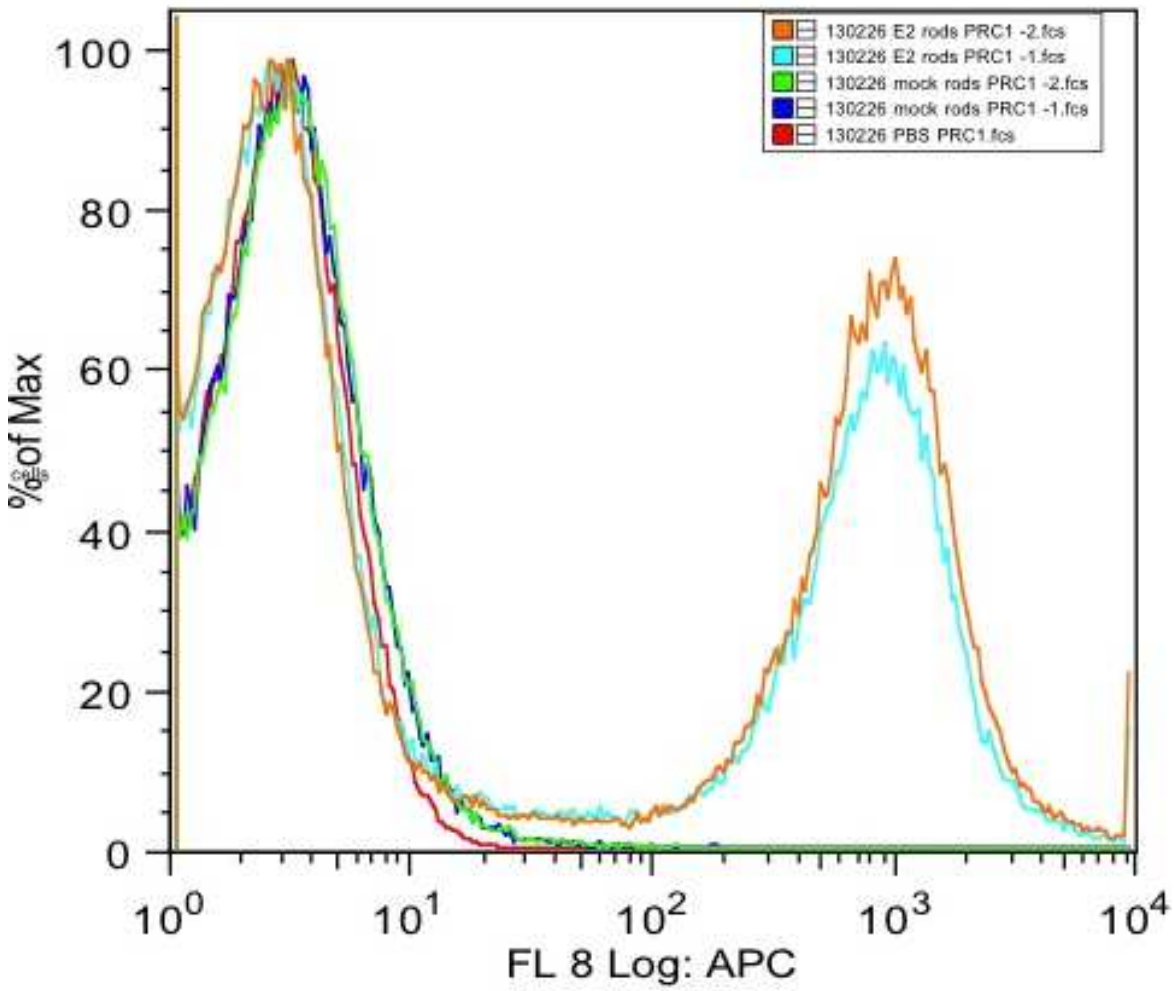


Figure A2.4. Histogram of fluorescent signal recovered from intraperitoneal (i.p.) wash on mice that received i.p. inoculation with either APC-conjugated E2 rods (orange and light blue) or APC-conjugated mock rods (green and dark blue). Mock rods were made from normal brain homogenate and were enriched with the same protocol as E2 rods. E2 rods show a strong peak in second and third decade, while mock rods remain in the range of background fluorescence

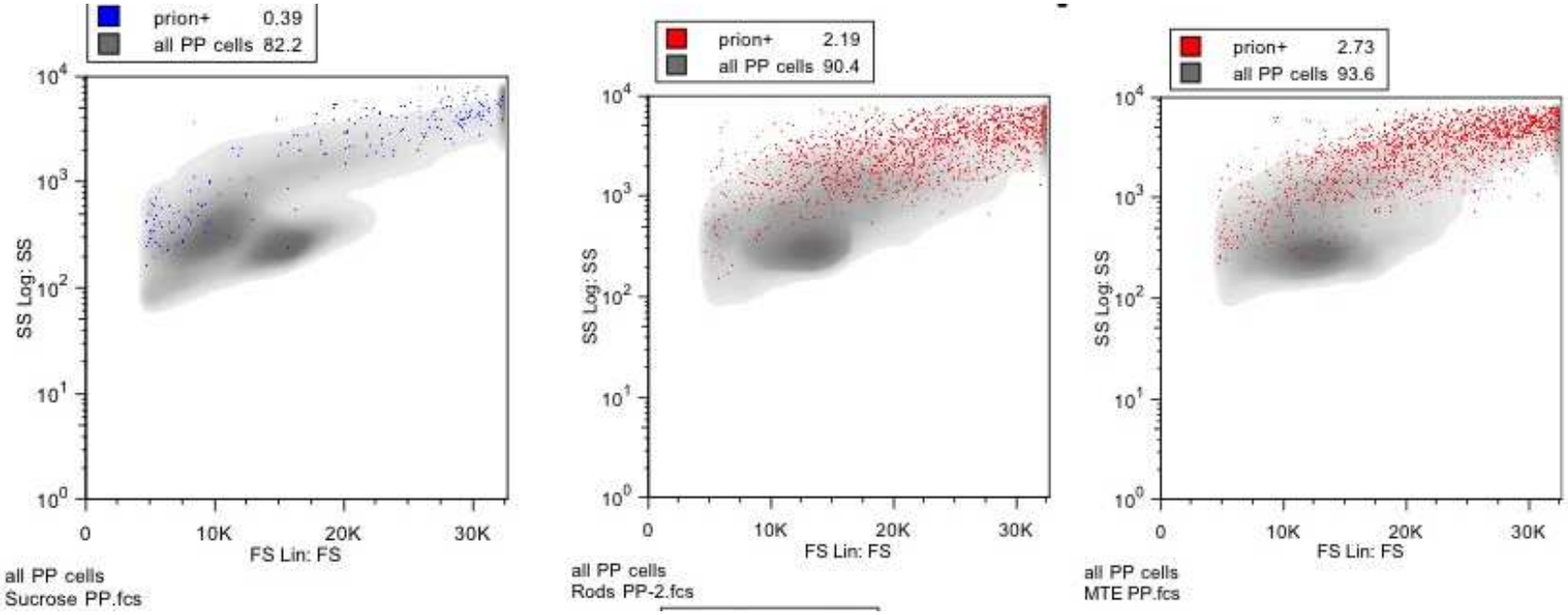


Figure A2.5. After 24 hours, strong fluorescent rods signal (highlighted in red) was recovered from the Peyer's Patches in both rods and rods-Mte treated animals.

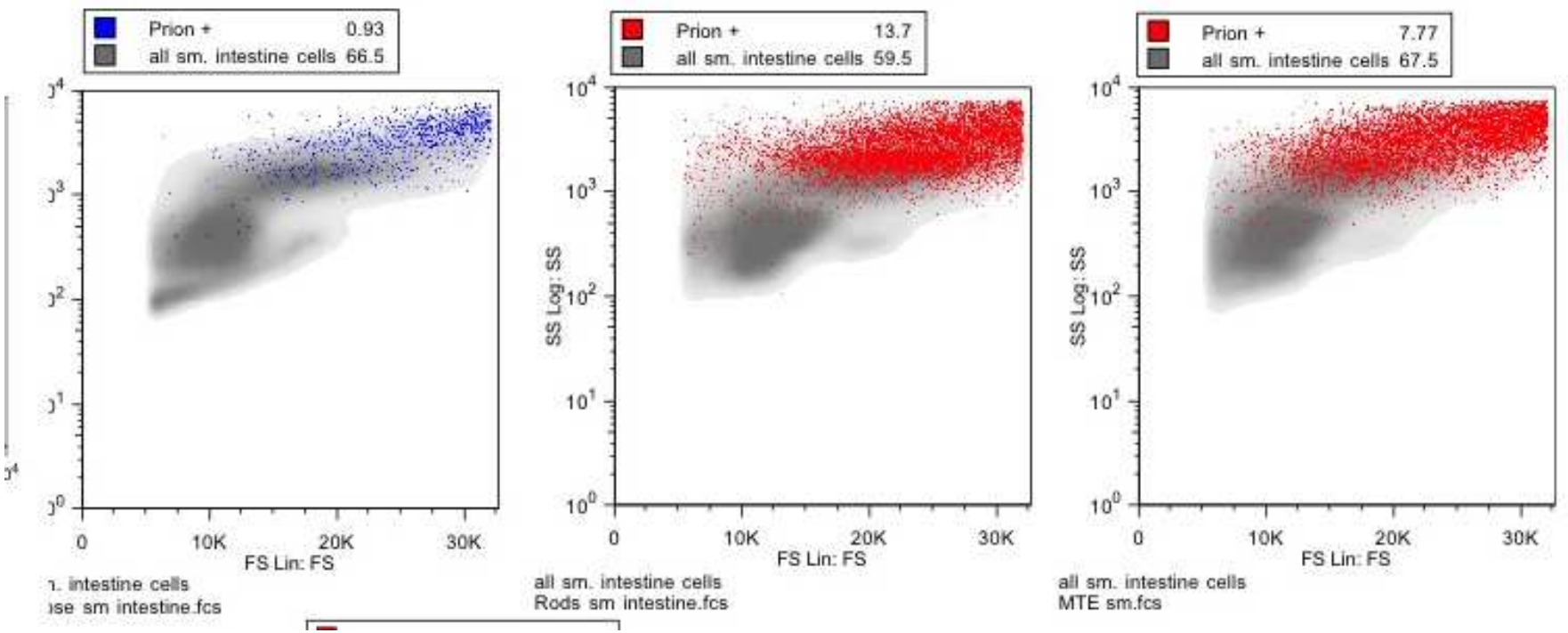
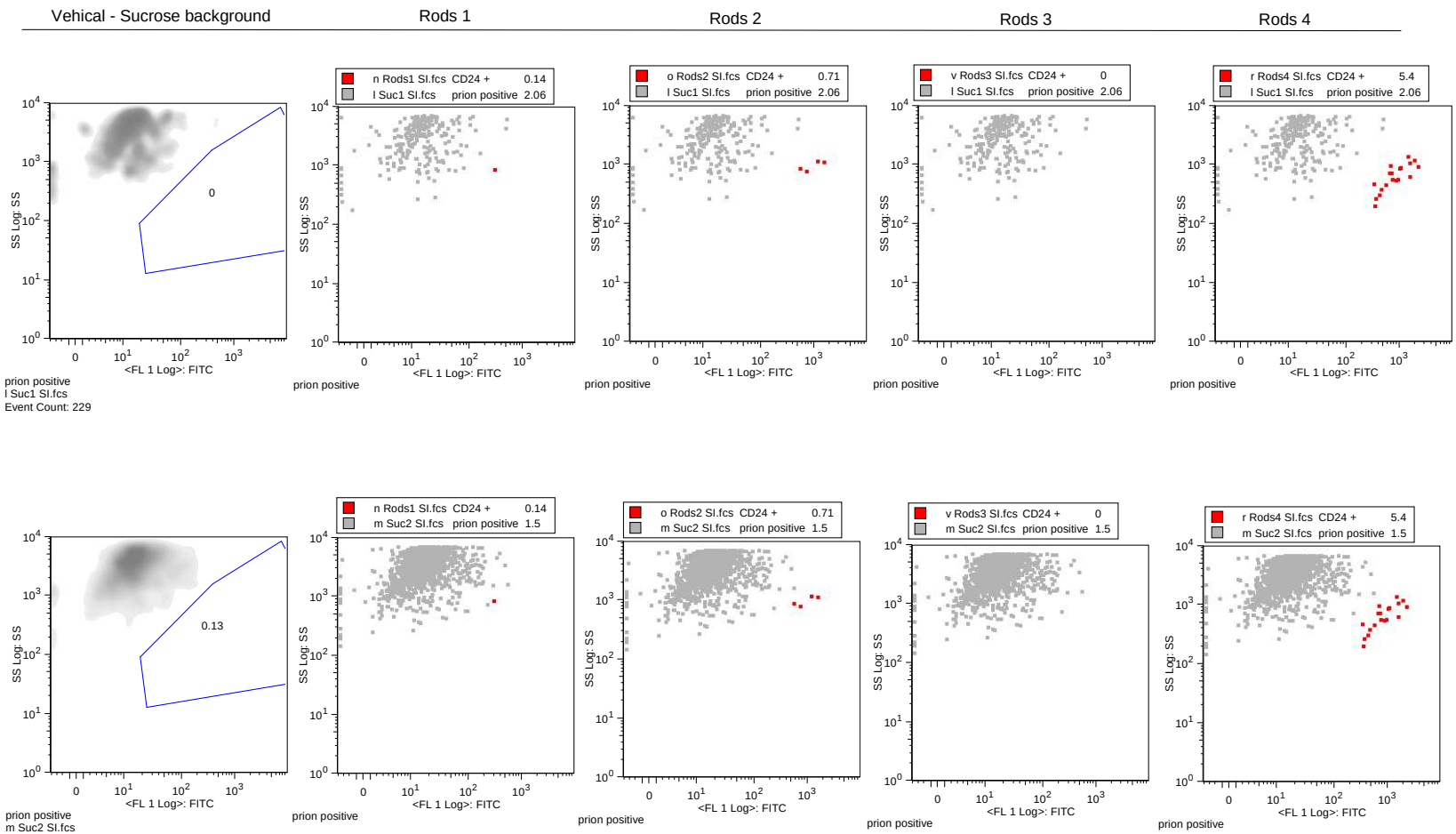


Figure A2.6. After 24 hours, strong fluorescent rods signal (highlighted in red) was also recovered from the small intestine in both rods and rods-Mte treated animals.

A. Epithelial Panel - CD24+ (FITC+)



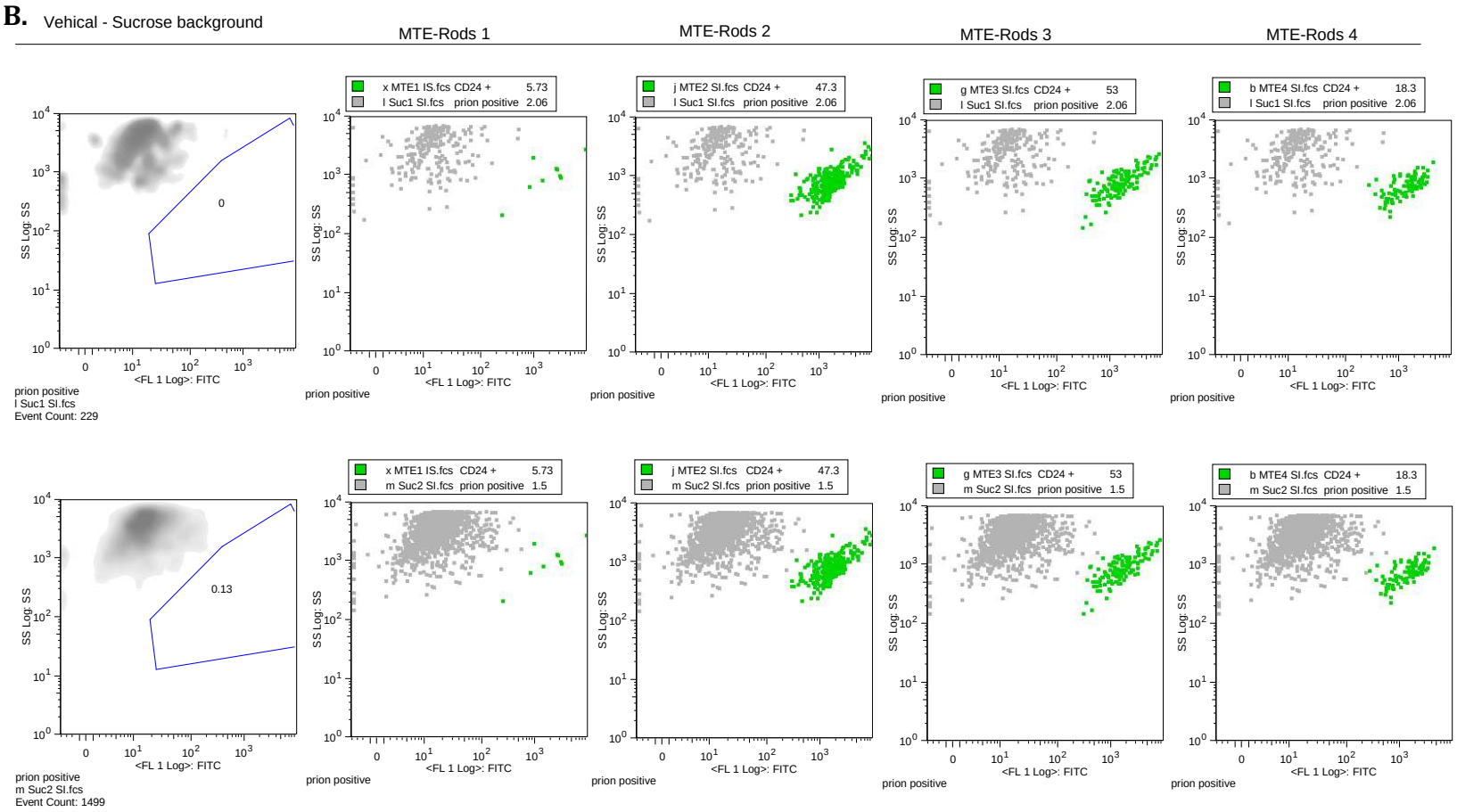


Figure A2.7. In an experiment looking at rods trafficking after 48 hours we found prion rod signal in the small intestine correlated with CD24+ expressing cells (highlighted in red A., and green B.), which are intestinal epithelial cells. This trend was most significant in the mice treated with Rods + Mte. Intestinal epithelial cells have been implicated in prion trafficking in a previous study. It is unclear why the presence of Mte changes this trafficking pattern.