DISSERTATION

SERIAL PROTEIN MISFOLDING CYCLIC AMPLIFCATION (sPMCA) TO DETECT SURROGATE MARKERS FOR CHRONIC WASTING DISEASE IN SURFACE WATER, MUNICIPAL WATER AND SOIL

Submitted by
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Graduate Degree Program in
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TRACY A. NICHOLS ENTITLED "THE USE OF PMCA TO DETECT SURROGATE MARKERS FOR CHRONIC WASTING DISEASE IN SURFACE WATER, MUNICIPAL DRINKING WATER AND SOIL" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

SERIAL PROTEIN MISFOLDING CYCLIC AMPLIFCATION (sPMCA) TO DETECT SURROGATE MARKERS FOR CHRONIC WASTING DISEASE IN SURFACE WATER, MUNICIPAL WATER AND SOIL

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy of deer and elk. Research has indicated that CWD is transmitted horizontally, and that both blood and saliva can transmit disease. Environmental exposure to pens where infected animals have been kept has resulted in disease transmission to deer. However, examination of environmental components such as soil and water for prions has been hampered by sensitivity limitations of conventional western blotting and inoculation limitations of bioassays. In this study we evaluated the ability of protein misfolding cyclic amplification (PMCA) to detect protease-resistant prion protein (PrP^{res}) in environmental samples such as water and soil. Serial protein misfolding cyclic amplification (sPMCA) of PrP^{res}, the misfolded proteinase-resistant protein associated with prion disease, was used to detect prion-infected brain homogenate spiked into soil and water to determine detection limits of this assay in environmental samples. The PrP^{res} detection limit for water after 6 rounds of PMCA was $1{:}26 \times 10^6$. Detection of a CWD spike in soil with our current methodology was not possible. We next evaluated surface and drinking water from a CWD endemic region of Colorado for PrP^{res} by sPMCA. PrP^{res} was detected in Cache la Poudre River and flocculant samples at a time of high snowmelt

runoff, suggesting that sPMCA can be a useful tool in evaluating water for PrP^{res} in CWD-endemic areas.

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DEDICATION

This dissertation is dedicated to my parents

Lester and Carol Nichols

My partner John Burt

and all of my friends who have seen me through this process;

Traci Allen, Mandy Kelley, Kelly Nesvacil, Cindy Pritekel and Kim Melville-Smith and

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Chapter 1

INTRODUCTION

Prion Diseases

Transmissible spongiform encephalopathies (TSEs) are an unusual group of infectious diseases that lead to progressive neurodegeneration and death. Included in this group are bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer, elk and moose and kuru and Creutzfeldt-Jakob disease in humans(23). These diseases are called prion diseases, after the term prion (proteinacous infectious only), which refers to an infectious proteinascious particle that can transmit disease in the absence of nucleic acids (47). Considerable evidence supports the hypothesis that the protease-resistant, misfolded prion protein (PrP^{Res}) is the causative agent in TSE (7, 19, 23, 30, 45).

The cellular prion protein (PrP^{C}) is a highly conserved, constitutively expressed glycoprotein encoded by a single gene found in many tissues (41, 58, 73). Structurally, the PrP^{C} protein is 231 amino acids in length with an unstructured N-terminus containing an octapeptide region (OR) that binds copper and a structured C-terminus containing 3 α-helices and 2 short β-sheets and two N-glycolsylation sites (fig 1.) (43). Glycans, composed of variable oligosacharidds are attached to the prion protein at amino acids 181 and/or 197 (fig. 1) (1, 53). However, the degree of glycosylation; unglycosylated, monoglycosylated or diglycosylated, can vary between species, tissues within the body and even regions within the brain (65). Addition of glycosylphosphatidylinositol (GPI) to

amino acid 231 anchors PrP^{C} in lipid rafts, areas of plasma membrane rich in cholesterol and sphingolipids that concentrate cell signaling molecules in the adjacent pericytoplasmic space. The function of PrP^{C} has been the subject of much research and debate. There are findings that support a cell signaling function, Exposure of PrP^{C} to the β-sheet rich conformer PrP^{Res} results in conversion, via a yet unidentified process, into more PrP^{Res}, which is characteristically resistant to proteinase- K (PK) digestion (45). The PK-resistant core is between 27 and 30 kD in weight and can been seen in the diagram in figure 1 (1). PK digestion of PrP^{Res} results in the removal of approximately 60 amino acids reducing the molecular weight 6 - 9 kD. This change in molecular weight gives rise to the characteristic shift of western blot bands, which distinguishes undigested and digested PK resistant PrP^{Res} (38). In contrast, normal PrP^{C} is monomeric and protease-sensitive (fig 2.) (45). The β-sheet rich nature of the PrP^{Res} predisposes it to aggregate into amyloid fibrils of various size which have a great deal of conformational stability (6, 18, 33, 69). The most infectious prion particle is a small 300-600 kDa oligomer that contains 14-28 prion protein monomers (64). Larger fibrils of PrP^{Res} have been shown to have little or no infectivity, suggesting that large aggregates sequester the infectious protein, reducing additional conversion.

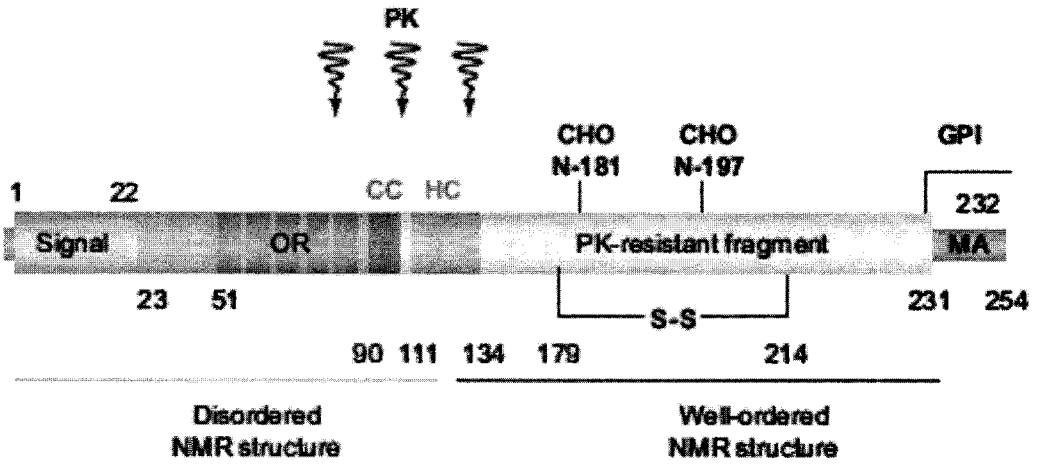


Fig. 1. The Prion Protein.
Numbers represent amino acid position. PK-resistant fragment persists after PK digestion. Arrows indicate proposed PK cleavage sites. OR- Octapeptide copper-binding region. CC- Charge cluster. HG- Hydrophobic core. S-S- Single disulfide bridge. MA- Membrane anchor region. GPI-Glycosyl phosphatidyl inositol. CHO- Facultative gycosylation sites. NMR- Nuclear magnetic resonance.. (Aguzzi et al., 2008)

Fig. 2. The Conformational shape of PrP^{C} and PrP^{Res}.
Structure on left represents the normal PrP^{C} protein conformation. Structure on right represents the aberrantly folded PrP^{Res}.

Chronic Wasting Disease

CWD affects deer, elk and moose, although not with equal infectivity, as deer are more susceptible than elk, which are more susceptible than moose. It is unclear why there is a difference between cervid species but it may be attributed to differences in eating and/or social behaviors (14, 51, 68). The incubation period of CWD can vary from 2 to 4 years, due in part to genetic polymorphisms, which have been shown to have a significant impact on incubation period and susceptibility to the disease (27, 38, 78). 95H, 96S, 132L and 225F polymorphisms protect elk against CWD, while 95Q, 96G, 132M and 225S

polymorphisms render elk highly susceptible (22, 27, 44). Disease susceptibility does not appear to be due to the amino acid itself, but rather the location, as 96S is protective, while 225S increases susceptibility in transgenic mice (38). Once clinical symptoms have begun death usually occurs within 4 months in captive situations and perhaps less in the wild where animals have a greater difficulty finding food and are exposed to low temperatures (78). CWD in deer and elk has no distinct clinical symptoms during the early and mid stage, however, during the terminal stage there are a number of clinical characteristics such as ataxia, head tremors, bruxism, excessive salivation caused by difficulty in swallowing, a lowered head and drooping ears, listlessness, hyper excitability, and polydipsia and polyurea in deer (78, 79). Terminal animals phenotypically have rough, dry coats, and are emaciated due to a complete loss of body fat (78). Death is often the result of aspiration pneumonia due to the loss of the swallowing reflex (78).

Unlike BSE, CWD has been shown to efficiently transmit horizontally (59), the exact mechanism of which remains the focus of numerous studies (36, 39). Indirect exposure via the environment may be a significant factor in transmission as saliva and blood have proven to be infectious, and current work suggests that urine and feces may also be a route of transmission (36, 39). Research indicates that prions are able to remain infectious in the environment and transmit disease years after contamination (9, 29, 52, 70). Both mineral and organic soil components, have been shown to strongly adsorb prion proteins (29, 48, 52, 61). Furthermore, soil adsorption of hamster scrapie has been shown to enhance the oral transmission of the disease by increasing the infectious titer by a factor of 680 when compared to the unbound hamster scrapie (28). In nature, decomposition of

CWD-positive carcasses, bodily fluids such as saliva and urine and feces all contact soil, which can be consumed during foraging and drinking (2, 26, 34-36, 39, 40, 67). Rainfall and snowmelt wash soil and organic materials into surface water (8). Soil components and organic carbon contribute to the total organic carbon (TOC) load in surface water. TOC is used as a surrogate marker for determining runoff intensity **(Pers. Comm** Kevin Gertig, (60)) and is composed of dissolved organic carbon (DOC), suspended particulate organic carbon (POC) and colloids (50). We **hypotheisize** that detection of PrP^{Res} would be more likely during times of high TOC levels. Shedding of PrP^{Res} in urine and feces into the environment might explain the efficient transmission of CWD in places such as Colorado where prevalence rates are high (76, 78). It is therefore likely that transmission in the wild is a combination of animal-to-animal and environmental exposures.

Domestic free-grazing animals such as cattle often occupy land that is also grazed by deer. To determine if cattle are susceptible to CWD, experiments were conducted in which twelve calves were intracerebrally inoculated with CWD from a deer. After 26 months all 12 lost weight and 11 developed clinical signs of prion disease. **Hisotpathology** revealed that although spongiform degeneration was not present, PK-resistant PrP was, indicating transmission of CWD (24). Although this study shows that domestic cattle are susceptible to CWD, the method in which they were exposed does not mimic that from a natural range setting. To address this issue a study was conducted in which the brains of 262 cattle that had grazed in a geographic location where CWD was endemic, were immunohistochemically evaluated for pathology indicative of prion disease. None of the brains exhibited neuropathological changes, suggesting that natural range exposure to CWD is not associated with TSE and pathology in cattle (21).

The susceptibility of humans to CWD is of obvious interest. Deer and elk from CWD endemic areas, such as Colorado, have been consumed for over 40 years and there does not appear to be an increase in human prions diseases such as Creutzfeldt-Jakob disease between the years of 1979 and 2001 (37). Additionally, transgenic humanized mice intracerebrally inoculated with elk CWD prions did not develop disease after 650 days post inoculation (dpi), while inoculated transgenic mice expressing elk PrP^C developed disease at 118 days dpi (31). In light of these studies it appears that there is a significant species barrier limiting CWD transmission to cattle and humans.

Chronic Wasting Disease in Fort Collins, Colorado

CWD is endemic to Northeastern Colorado and was first described in mule deer in 1967 in Fort Collins (79). The area surrounding Fort Collins has the highest incidence of CWD in Colorado (fig. 4) (76). Although more concentrated in this area, CWD has been found in deer (*Odocoileus hemionus and virginianus*) and elk (*Cervus elaphus nelsoni*) throughout Colorado, Wyoming, Montana, South Dakota and parts of Canada and deer in Minnesota, Wisconsin, Illinois, Oklahoma, New York, Kansas and Nebraska (4, 78). The Colorado Division of Wildlife (CDoW) has conducted considerable research on CWD both in the wild and at its research facility in Fort Collins, CO. This research facility lies several hundred meters north of the city of Fort Collins water treatment plant (fig. 3) . The potential adsorption of CWD prions to dust particles from the **CDoW** research facility and subsequent movement onto land occupied by the water treatment plant was the catalyst for the research in this dissertation.

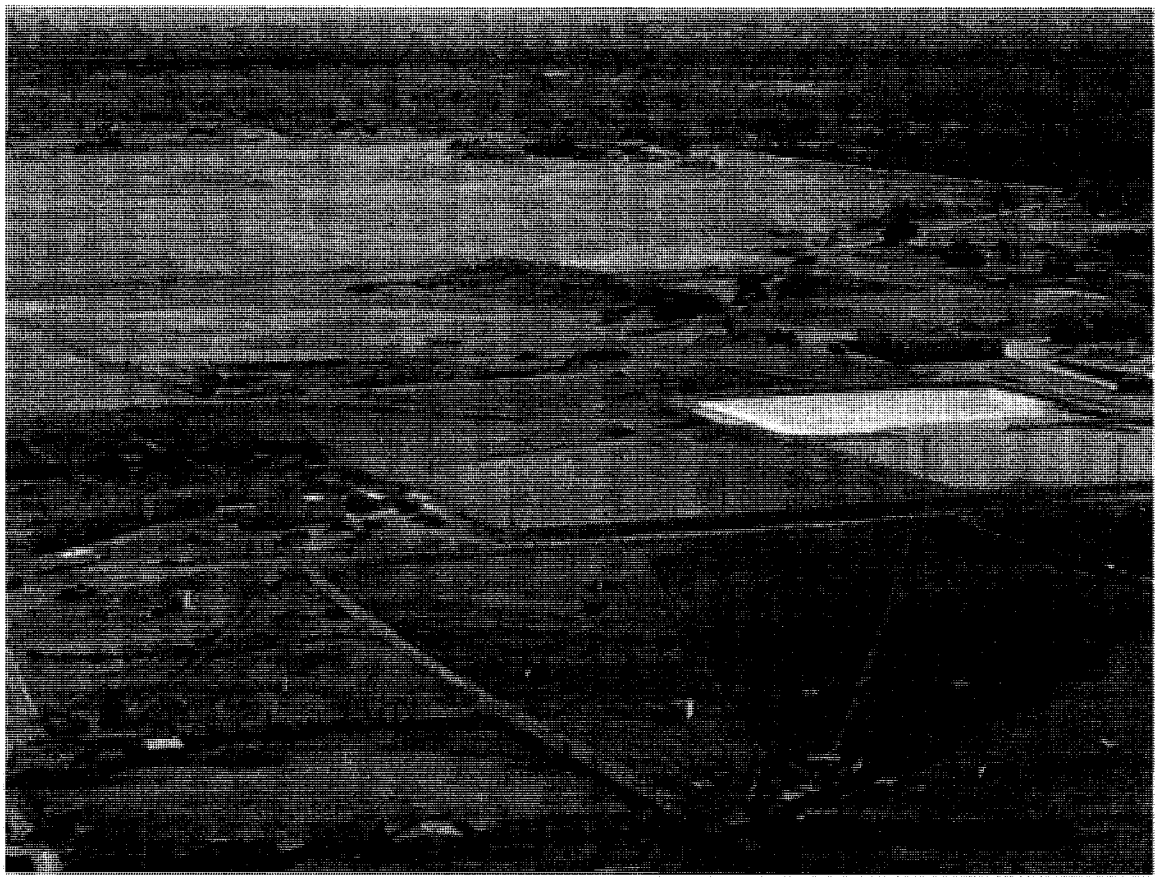


Fig. 3. Colorado Division of Wildlife research site and the Fort Collins H2O treatment plant.
The DOW facility is in the foreground with the water treatment plant filter backwash retention ponds to the right. Photo courtesy of the City of Fort Collins.

However, the city of Fort Collins lies along the foothills of the Rocky Mountains in Northeastern Colorado and relies on surface water from the western slope of the Colorado Rockies and more locally from the Cache la Poudre River, which is part of the South Platte River basin (fig 5), an area known to have a high incidence of CWD (76). Water from the western slope is transported across the continental divide and stored in reservoirs, initially in Estes Park, Colorado and then in Horsetooth Reservoir above the city of Fort Collins. Although both sources are used for drinking water, the ratio varies based on the time of year (pers. comm. Kevin Gertig, City of Fort Collins water

treatment). In spring and summer a higher percentage of Cache la Poudre River water is used and in the fall and winter Horsetooth water predominates. Considering the high number of infected cervids in the South Platte River Basin and possibility of environmental CWD contamination, it is conceivable that these surface water sources could be contaminated with minute quantities of CWD.

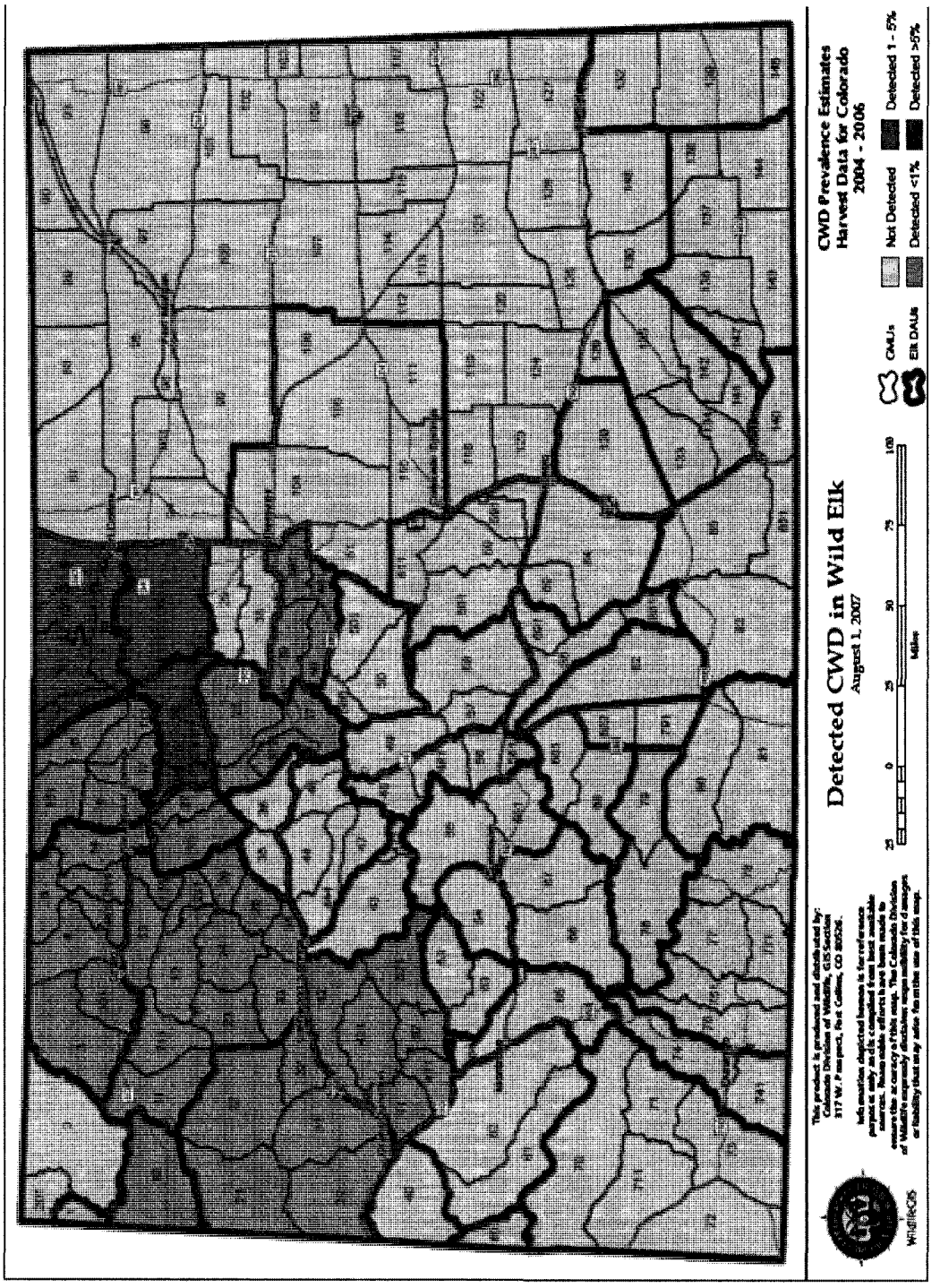


Fig. 4. Colorado Division of Wildlife elk CWD Occurrence Map

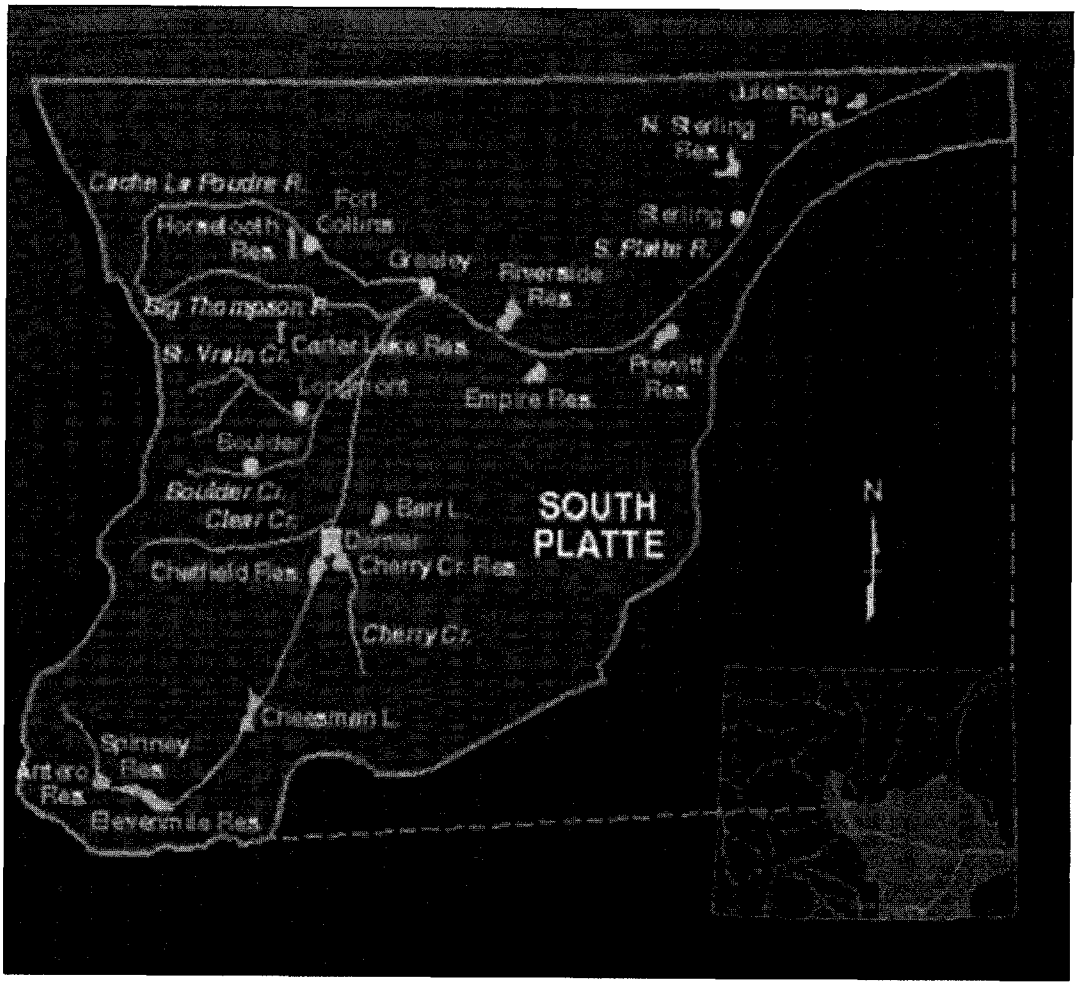


Fig 5. The South Platte River Basin
The Colorado Division of Wildlife CWD occurrence map and the South Platte River basin watershed map have a great deal of overlap.

Diagnosing Prion Diseases

Standard CWD diagnostic tests include histology and immunohistochemistry, which utilizes acid hydrolysis and prion-specific antibody staining to examine tissues for hallmarks of prion diseases. PrP^{C} -specific antibodies detect PrP^{Res} deposition and Hematoxylin and eosin staining reveal spongiform vacuolation characteristic of neuronal degeneration (78). Spongiform vacuolation and PrP^{Res} deposition are often not co-localized and PrP^{Res} deposition can be seen in asymptomatic animals while vacuolation appears when clinical symptoms become evident (67, 78). Although PrP^{Res} deposition is common in lymphoid tissue, there are no spongiform lesions in these areas (42, 49, 63, 78). PrP^{Res} can be detected in wide variety of tissues such as tonsils, spleen, retina, skeletal muscle, peripheral nervous system, endocrine organs, blood, saliva, lymph nodes, spinal cord and most strongly in the brain (2, 36, 78). PrP^{Res} amyloid fibrils and plaques are an indicator of prion disease, although evidence is mounting that suggests that these fibrils are not the cytotoxic species (13, 18, 77). Another hallmark of prion infected brain is the presence of astrogliosis, an increase in the proliferation of astrocytes, which can be detected by the presence of glial fibrillary acidic protein (GFAP) (16). Although histology is a very useful tool, it is limited to the evaluation of animal tissues, often postmortem.

The standard biochemical technique for detecting PrP^{Res} protein in tissues is western blotting following digestion with proteinase K (PK). PrP^{C} is sensitive to PK digestion, while the infectious core of PrP^{Res} is not and can therefore be seen on a western blot (46). Western blotting has been extensively and successfully used to look for the presence of

PrP^{Res} in brain, muscle, urine and blood in humans, mice, hamsters, cattle, sheep and cervids (2, 26, 40, 62, 70, 71)

Protein Misfolding Cyclic Amplification (PMCA)

The detection of small amounts of Prp^{res} is quite limited due to the low sensitivity of commonly used methods such as Western blotting and immunohistochemistry that prohibits the detection of small amounts of infectious prions in animal tissues and environmental samples. Protein misfolding cyclic amplification (PMCA) is an *in vitro* technique that enables the amplification of minute amounts of PrP^{res} by rapidly converting PrP^{c} to more PrP^{res} (11, 54, 56, 66). Initial PMCA experiments were conducted utilizing hamster-adapted scrapie strain 263 K (56). Brain homogenate is generated from an uninfected animal and is used as the source of PrP^{c} substrate. Incubation of PrP^{c} with a relatively small amount of PrP^{res} converts the PrP^{c} to PrP^{res}, increasing its concentration to a level that can be detected by conventional western blotting. As mentioned previously, infectious prion proteins aggregate into fibrils that appear to slow conversion of PrP^{c} to PrP^{res} (18). To successfully generate PrP^{res}, these aggregates must be disrupted to create smaller, more numerous "seeds" which can then continue to convert PrP^{c}. In PMCA this is accomplished by the use of a sonication step that disrupts prion aggregates (fig. 6). To further increase the sensitivity of PMCA, serial PMCA (sPMCA) was developed (5). This modification, which entails re-diluting PMCA samples into fresh brain homogenate after a specific number of PMCA cycles, increased amplification with each PMCA round (5). Castilla *et.al.* have shown that sPMCA gives a 10^{7}-fold increase in sensitivity after seven PMCA rounds compared to standard detection methods and is able to able to detect as few as 8,000 molecules of scrapie PrP^{res} (12).

sPMCA has been used to successfully amplify PrP^{res} from BSE infected cows, scrapie-infected mice, goats and sheep, CJD afflicted humans and CWD-infected deer and elk (32, 66).

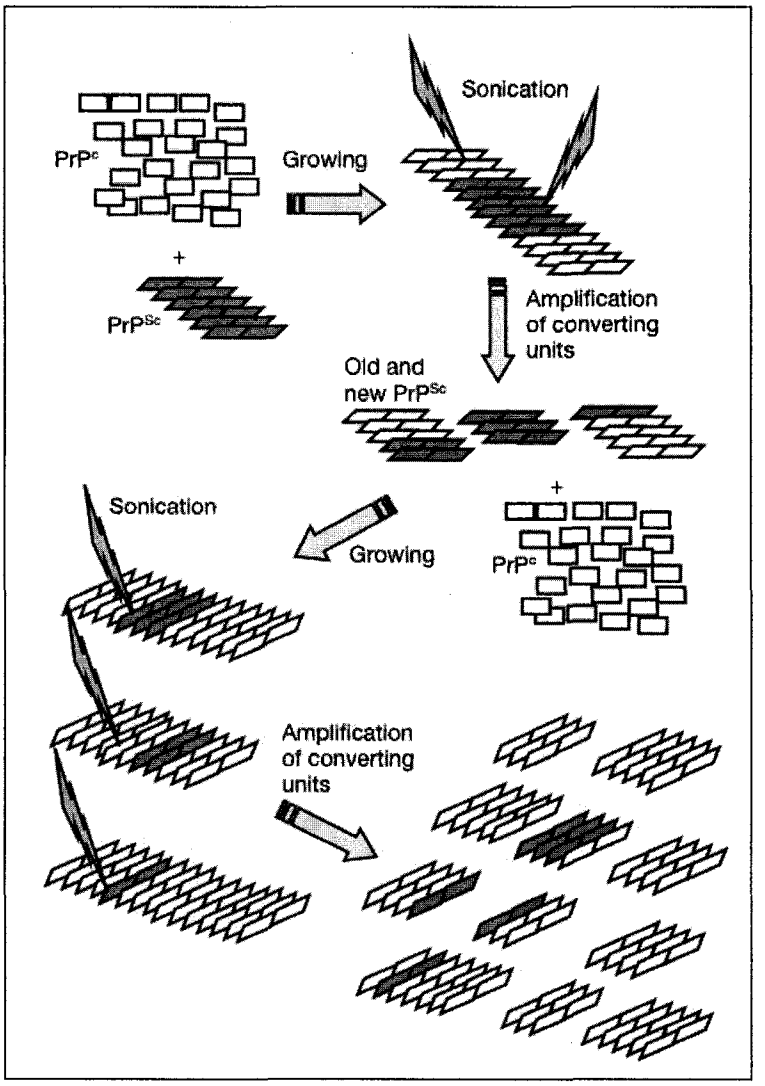


Fig. 6 . PMCA Concept. Saborio et al. 2001 (17).

Can sPMCA be used to detect small amounts of PrP^{res} in environmental samples such as soil and water? Can PMCA detect PrP^{res} in water entering and leaving the Fort Collins water treatment plant? The work in this dissertation addresses these questions. Although

there does not appear to be cross-species transmission of CWD to humans (4, 31, 37, 80), it is important to determine if the regional population is exposed to CWD prions in the drinking water and to better understand the movement of CWD in the environment.

Spontaneous generation

The sPMCA assay has great sensitivity, but it also possesses the ability to generate *de novo* PrP^{res}. Deleault *et al.* have found that using purified and copurified lipids in the presence of polyanions that PrP^{res} was formed during sPMCA. These *in vitro* generated PrP^{res} molecules were able to cause transmissible disease in hamsters (17). Atarashi *et al.* were also able to generate spontaneous PrP^{res} from recombinant mouse PrP^{C} using PMCA, although the size of the core after PK digestion varied from that of seeded PrP^{res} (3).

Chapter 2

DETECTION OF PROTEASE-RESISTANT PRION PROTEIN IN WATER FROM A CWD-ENDEMIC AREA

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are an unusual group of infectious diseases that lead to progressive neurodegeneration and death. Included in this group are bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer, elk and moose and kuru and Creutzfeldt-Jakob disease in humans (23). Considerable evidence supports the hypothesis that the protease-resistant misfolded prion protein (PrP^{Res}) is the causative agent in TSEs (7, 19, 23, 30, 45). Exposure of PrP^{C} to β-sheet rich conformer results in conversion, via a yet unidentified process, into more PrP^{Res}, which is characteristically resistant to proteinase- K (PK) digestion (45). PrP^{Res} can be detected in wide variety of tissues such as tonsils, spleen, retina, skeletal muscle, peripheral nervous system, endocrine organs, blood, saliva, lymph nodes, spinal cord and most strongly in the brain (2, 26, 36, 67, 78).

Chronic wasting disease (CWD) is endemic to Northeastern Colorado and was first described in mule deer in 1967 in Fort Collins (79). The area surrounding Fort Collins has the highest incidence of CWD in Colorado (76), but positive deer (*Odocoileus hemionus and virginianus*) and elk (*Cervus elaphus nelsoni*) have been found throughout Colorado, Wyoming, Montana, Minnesota, Wisconsin, Oklahoma, New York, Kansas,

Nebraska, South Dakota and parts of Canada (78). The Colorado Division of Wildlife (CDoW) has conducted considerable research on CWD both in the wild and at its research facility in Fort Collins, CO. This research facility lies adjacent several hundred meters south of the city of Fort Collins water treatment plant. The potential adsorption of CWD on dust particles from the CDoW research facility and subsequent movement onto land occupied by the water treatment plant was the catalyst for this research. The city of Fort Collins lies along the foothills of the Rocky Mountains in Northeastern Colorado and relies on surface water from the western slope of the Colorado Rockies and more locally from the Cache la Poudre River, which is part of the South Platte River basin, as its source for drinking water (pers. comm. Kevin Gertig). Considering the high number of infected cervids in the South Platte River Basin, and the possibility of environmental CWD contamination, it is conceivable that these surface water sources could be contaminated with minute quantities of CWD prions.

The detection of small amounts of Prp^{res} is quite limited due to the low of sensitivity of commonly used detection methods such as Western Blotting and immunohistochemistry. This lack of sensitivity, therefore, prohibits the detection of small amounts of infectious prions in animal tissues and environmental samples by these methods. Protein misfolding cyclic amplification (PMCA) is an *in vitro* technique that enables the amplification of minute amounts of PrP^{res} by allowing the rapid conversion of PrP^{c} to PrP^{res} (12, 55, 56, 66). Initial PMCA experiments were conducted utilizing hamster-adapted scrapie strain 263 K (56). Brain homogenate is generated from an uninfected animal and is used as the source of PrP^{c} substrate. The unknown sample is then added, which if positive, will begin to convert the PrP^{c} to PrP^{res}, increasing its

concentration to a level that can be detected by conventional western blotting. As mentioned previously, infectious prion proteins aggregate into fibrils that appear to slow conversion of PrP^{c} to PrP^{res} (18). To successfully generate PrP^{res}, these aggregates must be disrupted to create smaller "seeds" which can then continue to convert PrP^{c}. In PMCA this is accomplished by the use of a sonication step that disrupts prion aggregates (fig. 6). To determine if the newly formed PrP^{res} possessed the ability to continue to convert more PrP^{C} to PrP^{res}, serial PMCA (sPMCA) was developed (5). This modification, which implements the placement of sample into fresh brain homogenate after a specific number of PMCA cycles, increased amplification with each sPMCA round (5). Castilla et.al. have shown that sPMCA gives a 10^{7}-fold increase in sensitivity when compared to standard detection methods and is able to able to detect as few as 8,000 molecules of scrapie PrP^{res} (12). sPMCA has been used to successfully amplify BSE from cows, scrapie from mice, goats and sheep, CJD and variant CJD from humans and CWD from deer and elk (32, 66).

The PMCA technique was utilized in this study to examine raw surface water and water from the Fort Collins water treatment plant for PrP^{res}. Although PMCA has been shown to be effective at amplifying CWD prions (32), the detection limit for CWD prions in water samples was necessary for the completion of this study. Using known CWD positive brain we established our detection limit in water, then analyzed raw Horsetooth Reservoir and Cache la Poudre River water entering the Fort Collins water treatment plant, water from all stages of processing and finished water. Various commercially

available bottled waters and Midwestern municipal water samples from states not known to have CWD were analyzed as controls.

Four stages of processing were examined; flocculation, sedimentation, filtration, and filter backwash water. We theorized that the flocculation step, in which alum is added, would be the most likely area to detect PrP^{res} as alum neutralizes the charge on dissolved particulate matter such as colloids and organic carbon, causing them to clump into a precipitate called flocculant, which settles out in the sedimentation step (fig. 7). After the flocculant sediments, clear water is skimmed off the top and continues to be filtered. Water was collected after sedimentation and filtration. We also analyzed finished water used to clean the sand filters that is forced backwards through the filters and stored in a filter backwash pond.

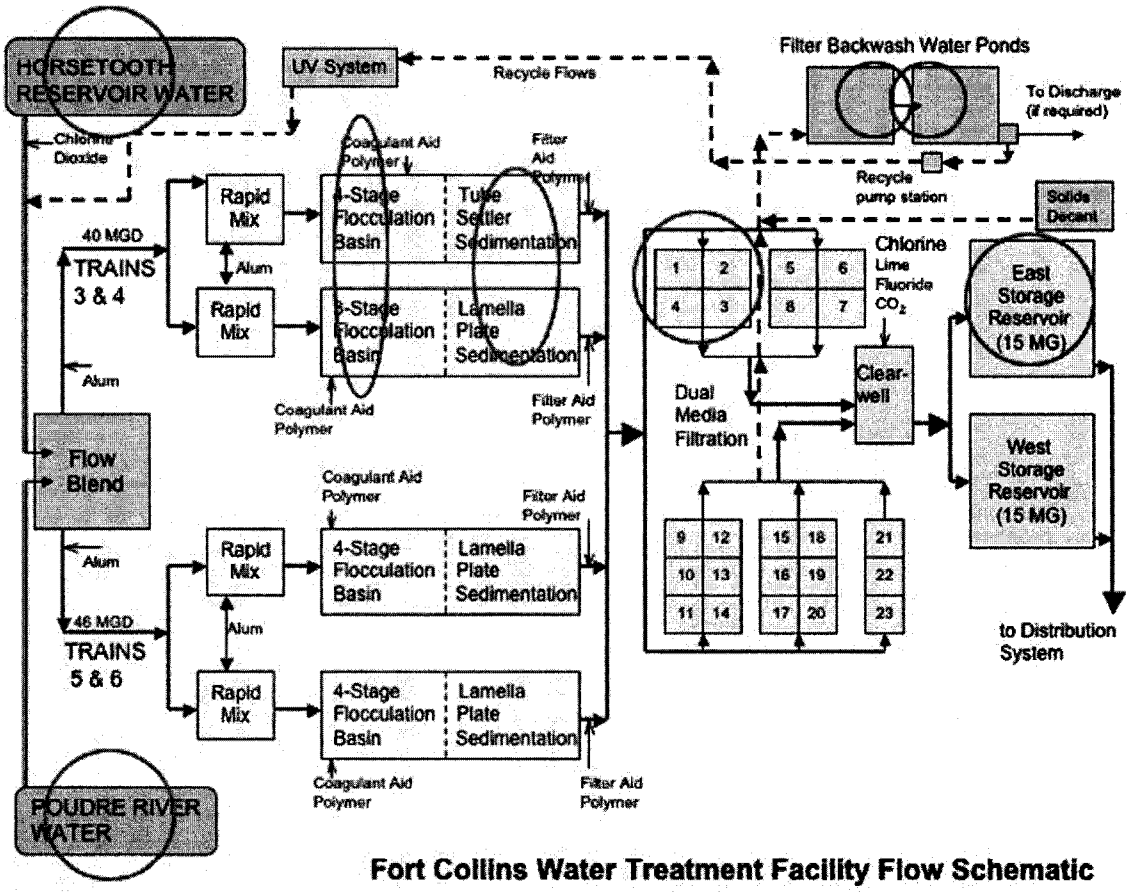


Fig. 7. Flow Schematic and Sample Collection Sites. **Circles represent where water samples were collected.**

Our data indicate that the Cache la Poudre River and flocculant contains minute quantities of PrP^{res} at times of high runoff, which can be amplified by sPMCA. Since PrP^{res} was detected from raw water samples collected from the water shed above the CDoW research area, it is likely that the PrP^{res} detected in water samples entering the water treatment plant derives from the watershed and not the CDoW facility. These results also indicate that PMCA may be a useful tool in determining the presence of prions in the environment.

2. Methods and Materials

2.1 Mice

Tg(cerPrP)5037 mice were generated in the Telling laboratory in a similar fashion to Tg1536 (10) by injection of fertilized FVB/ PrP null oocytes with the MoPrPXho expression vector and resulting in a wider tissue distribution of CerPrP expression that was increased to 6 times the normal expression of MoPrP in wild type mice. Mice were housed and maintained at Lab Animal Resources, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University.

2.2 Preparation of brain homogenates

Tg5037 mice were perfused with 30 ml of 1x phosphate buffered saline (PBS) plus 5 mM EDTA prior to removal of the brain. A 10% (wt/vol) normal brain homogenate (NBH) was prepared in PMCA conversion buffer (12). Briefly, brains were halved and snap frozen in liquid nitrogen. The halves were weighed and the appropriate volume of PMCA buffer 1 (1X PBS containing 150 mM NaCl, 4 mM EDTA) and complete Protease Inhibitor Cocktail (Roche)) was added to tubes with glass beads for FastPrep homogenizing (Biogene) to generate a 20% wt/vol solution. An equal volume of PMCA buffer 2 (PMCA 1 buffer without protease inhibitor plus 2.0% triton X-100) was added and samples were left to incubate on ice for 20 min then clarified by centrifugation at 1000 RPM for 5 minutes. The supernatant was carefully removed and frozen at -80° C.

2.3 sPMCA 96-well plate procedure

The sPMCA (serial protein misfolding cyclic amplification) technique used was based on that described by Saa et al, however, a number of modifications were made (55). Prion detection limits in water and evaluation of environmental samples were conducted by

adding 25µl of each sample to 25µl of NBH per well on a 96 well PCR plate. The plate was then subjected to 40 sec pulses of sonication at 37° C, power setting 7, every 30 min for 24 hr using a 3000MP sonicator (Misonix). After 24 hrs each sample was mixed and 25µl transferred to 25µl of fresh NBH. The plate was again sonicated as described above constituting 2 rounds of PMCA. This process was repeated 4 times for a total of six rounds.

2.4 sPMCA 200 µl tube procedure

25µl of each sample was added to 25µl of NBH in individual 200 µl PCR tubes sealed shut with parafilm^{TM}. The tubes were then subjected to 40 sec pulses of sonication at 37° C, power setting 7, every 30 min for 24 hr using a 3000MP sonicator (Misonix). After 24 hrs each sample was mixed and 25µl transferred to to 25µl of fresh NBH. The tubes were again sonicated as described above constituting 2 rounds of PMCA. This process was repeated 4 times for a total of six rounds. A postive amplification control of 1:100,000 CWD+ brain homogenate was amplified with each sample group.

2.5 CWD-spiked water detection

Deionized water samples were spiked with a 10% solution of CWD-positive brain made from a terminally sick deer (D10). 2-fold dilutions were made spanning 1:1600 through 1:422.4 x10^6 then amplified by sPMCA for 6 rounds.

2.6 Environmental water samples

Water samples were collected with the assistance of City of Fort Collins water treatment plant personnel. The following samples were collected in the plant: raw water (Cache la Poudre River and Horsetooth Reservoir), flocculation tank water, sediment tank water, post filtered water, finished water and backwash pond water. Additionally,

mountain water from the North fork of the Cache la Poudre River and from Halligan Reservoir was collected by plant personnel. After collection samples were frozen at -80° C.

2.7 Bottled water and Midwestern municipal water samples

Bottled water and municipal water samples were collected and analyzed from a wide range of sources. The following bottled water samples were evaluated: Electrolyte Enhanced deionized water (Whole Foods), Natural Spring Water bottled at Clairvic Spring in Volvic, France (Volvic) and Italian Sparkling Mineral Water, bottled in Silva Lucca, Italy (Whole Foods). Municipal drinking water was collected from Indiana, Michigan and Minnesota.

2.8 Ultrafiltration of water samples

An Anotop 10 Plus ultra filtration filter with a pore size of 20 nm (Whatman) was used to filter PrP^{res}-positive Cashe la Poudre River and flocculant samples collected on 5-22 -07 and 1:100,000 D10-spiked water samples.

2.9 PK digestion and Western blot

Amplified samples were treated with 50μg/ml of proteinase-K (Roche) for 30 min at 47° C and PK inactivated by the addition of lithium dodecyl sulfate loading buffer (Invitrogen) and heat inactivation for five minutes at 99° C. Samples were electrophoresed through 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen), transferred to Immobilon PVDF membrane (Millipore), blocked with 5% nonfat milk in 0.2% Tween 20 in PBS and probed with a 1:20,000 dilution of Bar 224/HRP-conjugated anti-PrP monoclonal antibody (SPI bio) in Superblock (Pierce). Western blots were incubated for five minutes with enhanced

chemiluminescent substrate (Millipore) and visualized using the FujiDoc gel documentation system (Fuji).

2.10 Mouse bioassay

Tg1536 mice were inoculated intracerebrally with 30µl of sonicated raw Cache la Poudre River and finished plant water supplemented with 100 units/mL Penicillin and 100 µg/mL Streptomycin (Gibco) in PBS prior to intracerebral inoculaions. Soncation and antibiotics were used to destroy microorganisms present in the water. A third group of mice was exposed chronically to $1x10^6$ D10 in drinking water to mimic environmental levels. Mice were monitored for clinical symptoms such as tail rigidity, impaired extensor reflex, akinesia, tremors, ataxia and paralysis. Mice exhibiting any 4 of these symptoms are considered terminally sick, euthanized and evaluated for CWD immunohistochemically.

3. Results

3.1 sPMCA 96-well plate analysis

After the completion of the above experiments, it was discovered that samples were migrating between wells in the 96-well plate causing wide-spread contamination (appendix B). This discovery necessitated the experiment to be repeated in its entirety.

3.2 sPMCA individual 200µl tubes

Due to the contamination experienced using the plate method, individual 200 µl PCR sample tubes sealed shut with parafilm^{TM} were used to repeat the experiments. Contamination was virtually eliminated. One hundred and fifteen known negative NBH, bottled water and Midwest water controls were evaluated for occurrence of PrP^{res}

spontaneous signal. One out 115 samples contained spontaneously generated PrP^{res} material, yielding a specificity of 99.2% (data not shown).

3.2 CWD spike detection limit in deionized water

It has been established that PrP^{Res} from CWD-infected brain material can be amplified using sPMCA (32). To determine the ability of sPMCA to amplify PrP^{Res} from CWD positive material serially diluted in water, we performed six, 24 hour rounds of PMCA on serial 2-fold D10 dilutions ranging from $1:1.6x10^6$ to $1:4.224\ x10^8$. After six rounds of PMCA our detection limit was$1:1.32x10^7$ (fig. 9) demonstrating the ability of sPMCA to significantly amplify minute amounts of PrP^{Res} from water. Without the use of sPMCA amplification, the greatest detectable dilution was 1:400 (fig. 8).

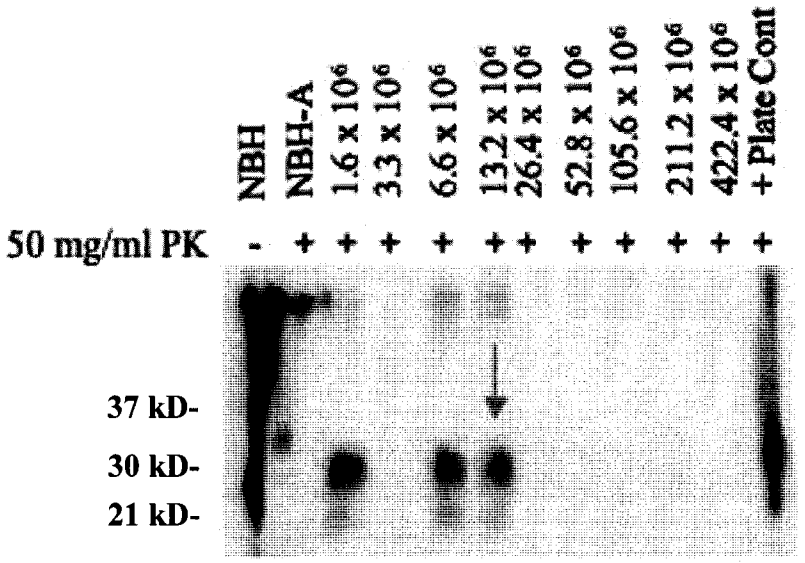


Fig. 8. sPMCA on 2-fold D10 dilutions 1:1600 – 1:422.4 x10^6. PK-proteinase K. NBH-Normal brain homogenate negative controls. Greatest detectable D10 concentration after 6 sPMCA rounds was 1:13.2 x 10^6 .

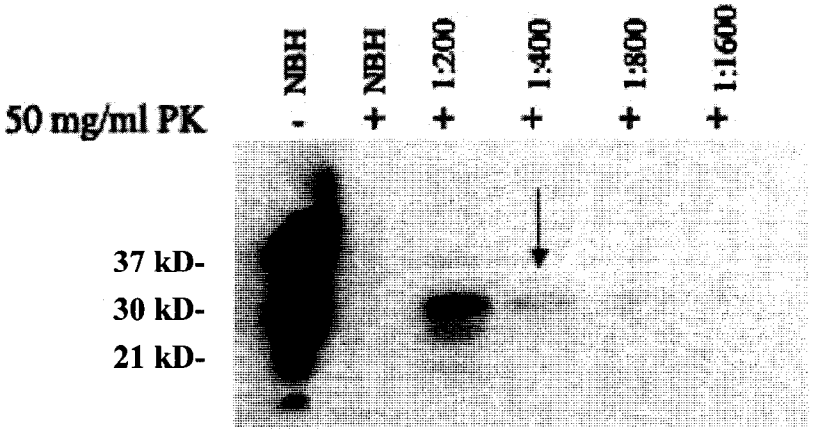


Fig. 9. Unamplified 2-fold D10 dilutions 1:200 - 1:1600.
PK-proteinase K. NBH-Normal brain homogenate negative control. The greatest detectable D10 dilution detected without the use of sPMCA was 1:400.

3.2 Evaluation of bottled water and municipal water samples from the Midwestern United States

To determine the specificity of PrP^{res} detection by sPMCA, we analyzed bottled and municipal water samples from areas where CWD has not been detected as additional negative controls. We failed to amplify PrP^{res} from bottled water samples from 3 different sources using sPMCA (fig. 10). Municipal water samples taken from 3 CWD-free Midwestern states; Indiana, Michigan and Minnesota, and subjected to 6 rounds of sPMCA were also negative for PrP^{res} (fig. 11).

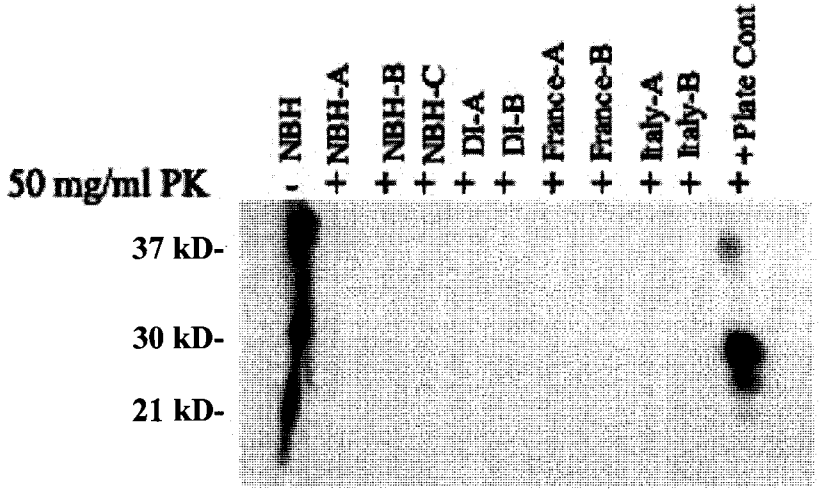


Fig 10. sPMCA of bottled water.
PK-proteinase K. D10- Unamplified 10% infected brain positive controls. NBH- Normal brain homogenate negative controls. D10+ -1:100,000 amplified positive amplification control. DI- Electrolyte-enhanced deionized water. FR- Natural spring water bottled in France. IT- Italian sparkling mineral water bottled in Italy. Figure represents one of three, 6 sPMCA round replicates executed. Each replicate was done in duplicate (A or B). All bottled water sample replicates were negative.

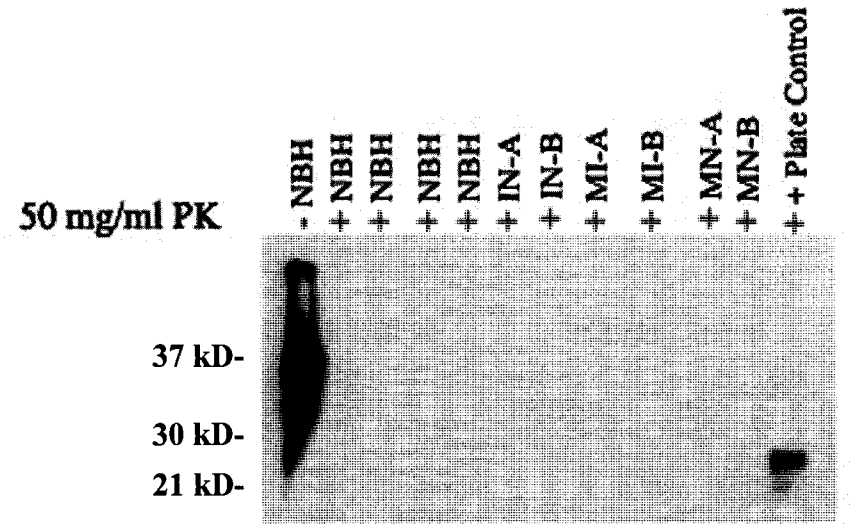


Fig 11. sPMCA of municipal water samples from the Midwestern United States.
PK-proteinase K. D10- Unamplified10% infected brain positive controls. NBH- Normal brain homogenate negative controls. D10+ -1:100,000 amplified positive amplification control. IN- Indiana. MI- Michigan. MN- Minnesota.. Figure represents one of three, 6 sPMCA round replicates executed. Each replicate was done in duplicate (A or B). All water sample replicates were negative.

3.3 Surface water collection and Fort Collins drinking water from various stages of processing

We next analyzed water samples from various stages of processing at the Fort Collins water treatment plant by sPMCA. Samples were collected with the aid of a City of Fort Collins water plant engineer at 4 different times of the year and amplified for 6 rounds of PMCA. Plant water samples were collected on 3-29-07, 5-10-07, 5-22-07, and 7-23-07. It was important to collect samples at different times of the year as the amount of snowmelt and consequently the amount of runoff, varies depending on the time of year.

Plant samples from 3-29-07, 5-10-07 and 7-23-07 were all negative for PrP^{res} (Fig. 12, 13, 14). However, plant samples collected on 5-22-07 displayed positive signal in Poudre River water and in water from the flocculation step (Fig. 15A). Each plant collection was replicated 3 times. Only samples in which positive signal was detected at least twice were considered positive. Replicates 2 and 3 can be viewed in appendix A.

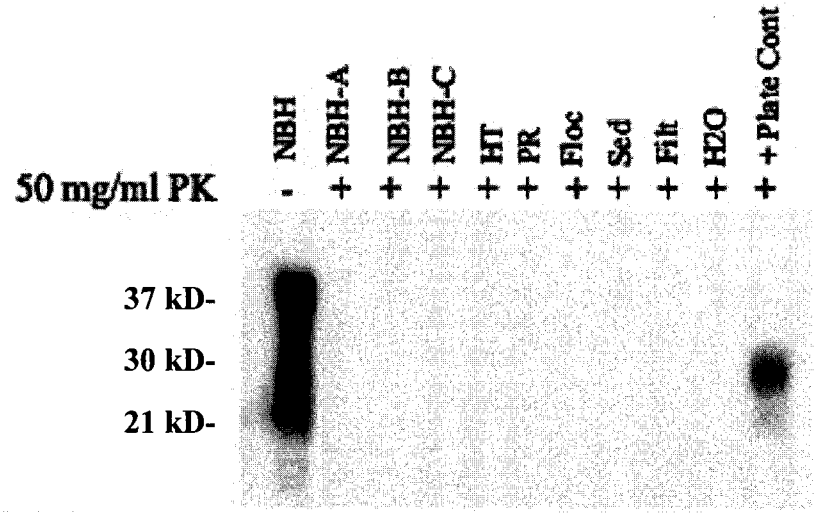


Fig. 12. 3-29-07 Plant sample.
PK-proteinase K. NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. + sPMCA control 1:100,000. This western blot is representative of 3 separate replicates after 6 sPMCA rounds. All samples were negative for PrP^{res}.

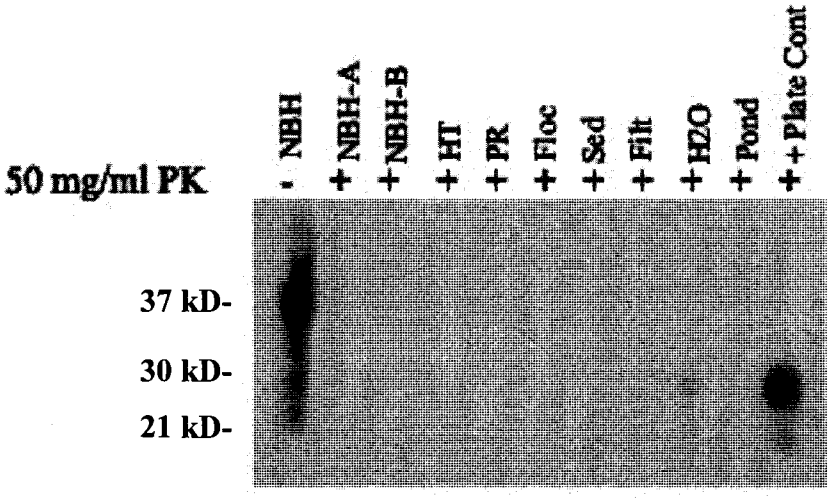


Fig. 13. 5-10-07 Plant sample.
PK-proteinase K. NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control 1:100,000. This western blot is representative of 3 separate replicates after 6 sPMCA rounds, each was done in duplicate. All samples were negative for PrP^{res}.

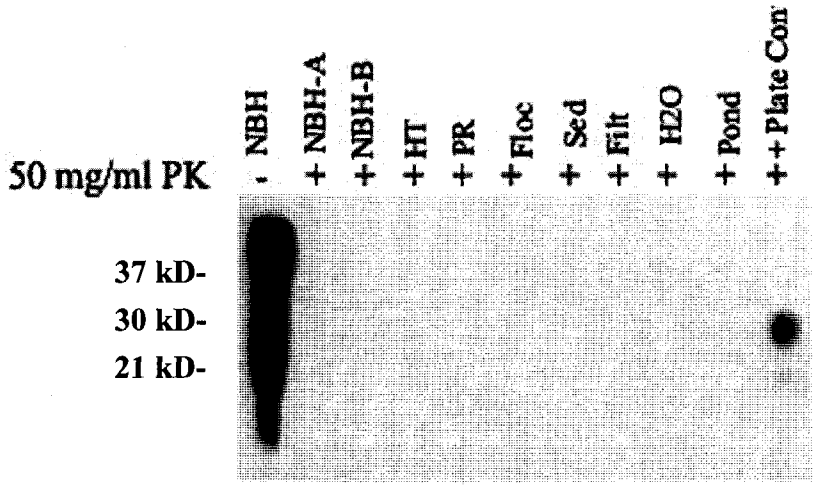
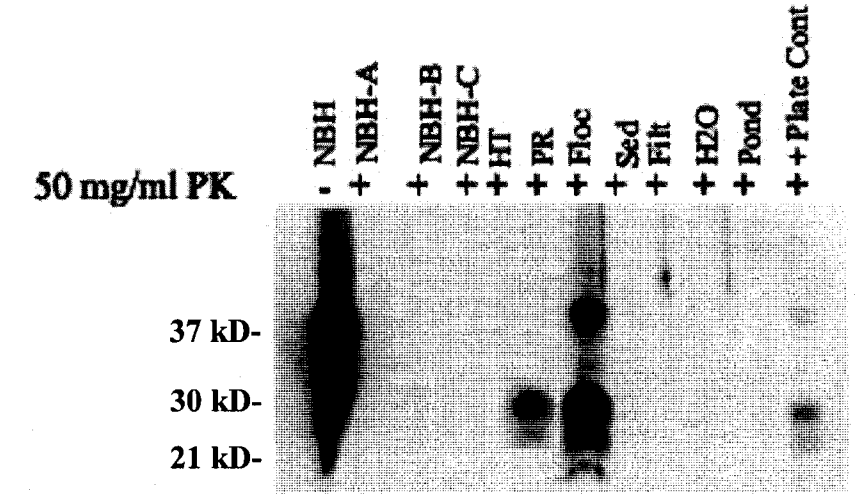


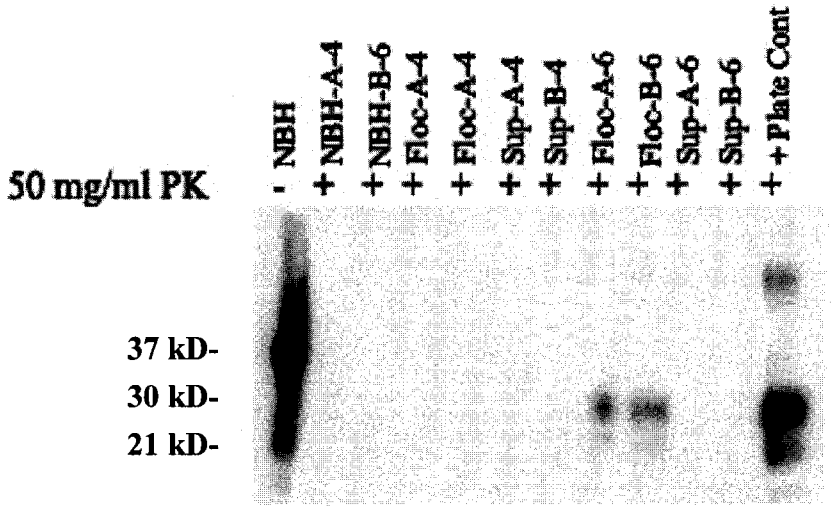
Fig. 14. 7-23-07 Plant sample.
PK-proteinase K. NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. Finished H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control 1:100,000. This western blot is representative of 3 separate replicates after 6 sPMCA rounds, each were done in duplicate. All samples were negative for PrP^{res}.

To determine which fraction of the flocculation water contained PrP^{res}, a simple experiment was conducted in which flocculant water was centrifuged separating the flocculant itself from the water. Figure 15B demonstrates that after 4 rounds of sPMCA no signal was detected in either fraction, however after 6 sPMCA rounds, PrP^{res} was detected exclusively in the flocculant fraction.

Raw water samples were collected by a City of Fort Collins engineer at two locations higher in the Cache la Poudre River watershed, Halligan reservoir and the north fork of the Cache la Poudre river on 5-11-07. After 6 rounds of sPMCA neither sample was positive for PrP^{res}, further supporting that PrP^{res} detection is dependent on time of year (Fig.16A and B).



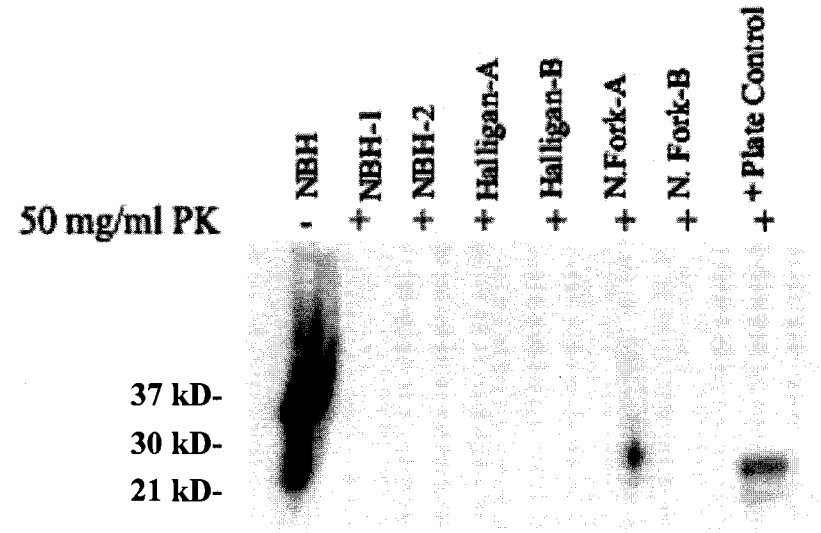
A.



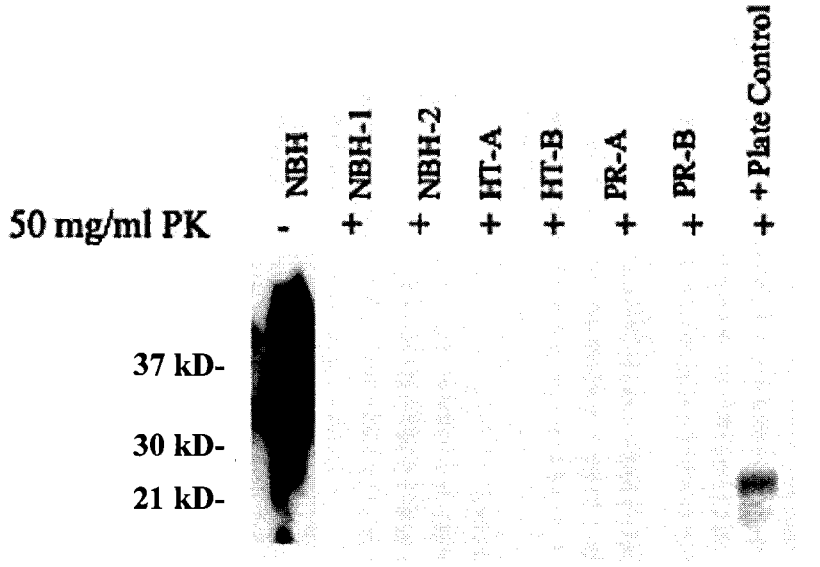
B.

Fig. 15. 5-22-07 Plant sample and flocculant analysis.

PK-proteinase K. NBH- Normal brain homogenate negative controls. **A**. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control 1:100,000. After 6 sPMCA roundsPrP^{res} was detected in Cache la Poudre River and floc.samples. **B**. Floc-precipitate fraction of flocculant tank sample Sup- water fraction of flocculant tank sample. Centrifuged flocculant water revealed that PrP^{res} was detectable only in the flocculant fraction and not the water fraction and only after 6 rounds of sPMCA.



A.



B

Figure 16. 5-11-07 Halligan Reservoir, north fork of the Cache la Poudre River.
PK-proteinase K. NBH- Normal brain homogenate negative controls. HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. **A**.Halligan and N.fork of samples collected on 5-11-07 were both negative for PrP^{res} after 6 sPMCA rounds. Due to the location of the dot in A , which is above that of the positive control, this sample was considered negative **B**. Horsetooth reservoir and Cache la Poudre river samples from 10-5-07 were also negative.

The amount of runoff, as measured by total organic carbon (TOC), positively correlates with detection of PrP^{res} in plant samples (Fig. 17). Positive samples collected on 5-22-07 were from a time of high snowmelt.

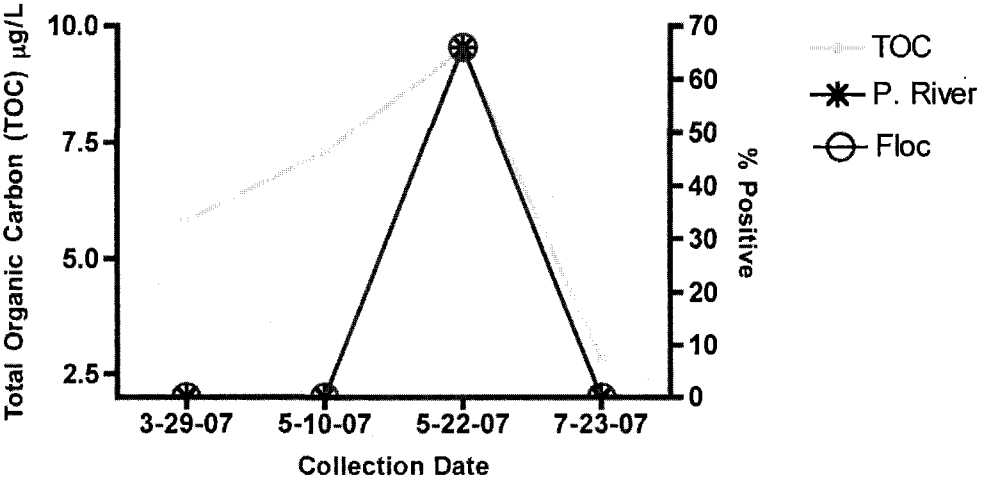


Fig. 17. Total Organic Carbon (TOC) and Percent Positive Left Y axis represents amount of TOC in µg/L. Right axis represents percent of positive City of Fort Collins drinking treatment plant samples after 6 sPMCA rounds. PrP^{res} positive signal was found on 5-22-07 in Cache la Poudre River and flocculant samples, which correlates to high runoff as measured by TOC levels.

3.4 Ultrafiltration of surface water samples and a CWD water spike

The most infectious PrP^{res} particle has been shown to contain14-28 molecules and has a size of approximately 17-27nm (64). In the body, PrP^{res} forms amyloid fibrils that are composed of many such oligomers (18).

PrP^{res} adsorbs to soil and organic carbon particles in the environment (15, 29, 48, 61). Adsorption would generate PrP^{res} particles larger in size than PrP^{res} alone. To determine whether PrP^{res} could be filtered from environmental samples, we passed them through a 20nm filter, the currently available technology in municipal ultrafiltration systems for drinking water treatment, then performed sPMCA on the filtrate. After 4 sPMCA rounds no signal was detected but after 6 sPMCA rounds signal began to emerge in the

1:100,000 positive control, illustrating that ultrafiltration can substantially reduce the amount of PrP^{res} in water samples (fig. 18).

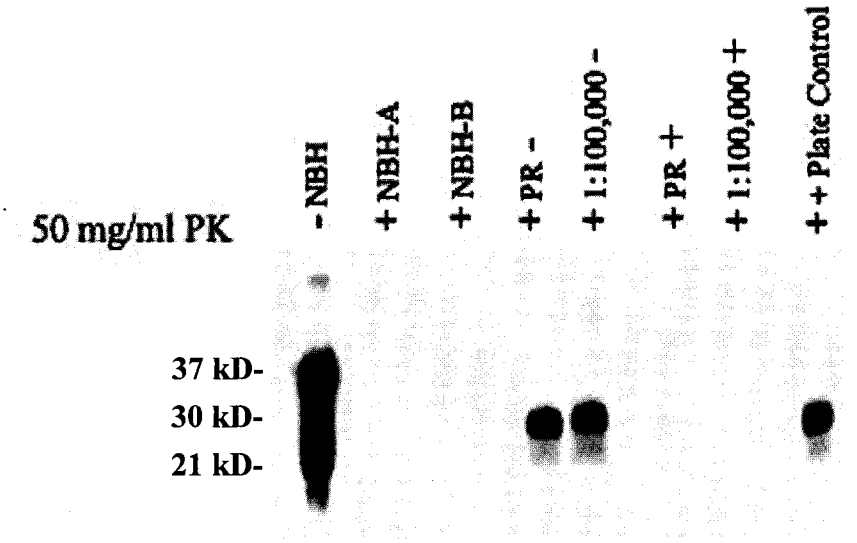


Figure 18. Ultrafiltration of PrP^{res} in water samples.PK-proteinase K. NBH- Normal brain homogenate negative controls. PR- -Cache la Poudre River water unfiltered. PR + -Cache la Poudre River water filtered with a 20 nm syringe filter. 1:100,000- - D10 dilution unfiltered. 1:100,000 + - D10 dilution filtered with a 20 nm syringe filter. + Plate Control – D10 1:100,000 . After 6 rounds of sPMCA filtered samples remained negative while unfiltered samples displayed a positive signal.

3.5 Mouse bioassay

To assess the infectivity of the positive material detected in the 5-22-07 plant collection, transgenic, cervidized mice were inoculated intracerebrally with Cache la Poudre River and finished tap water. Mice remain asymptomatic >400 days post inoculation (dpi) at the time of the writing of this paper. To assess the effects of chronic exposure of the transgenic mice to a small concentration of known infectious material,

mice were chronically exposed to a $1x10^6$ dose of CWD positive D10 brain in their drinking water (Fig. 19). This dose is approximately 13 times greater than that found in the water samples. These mice also remain asymptomatic > 400 dpi.

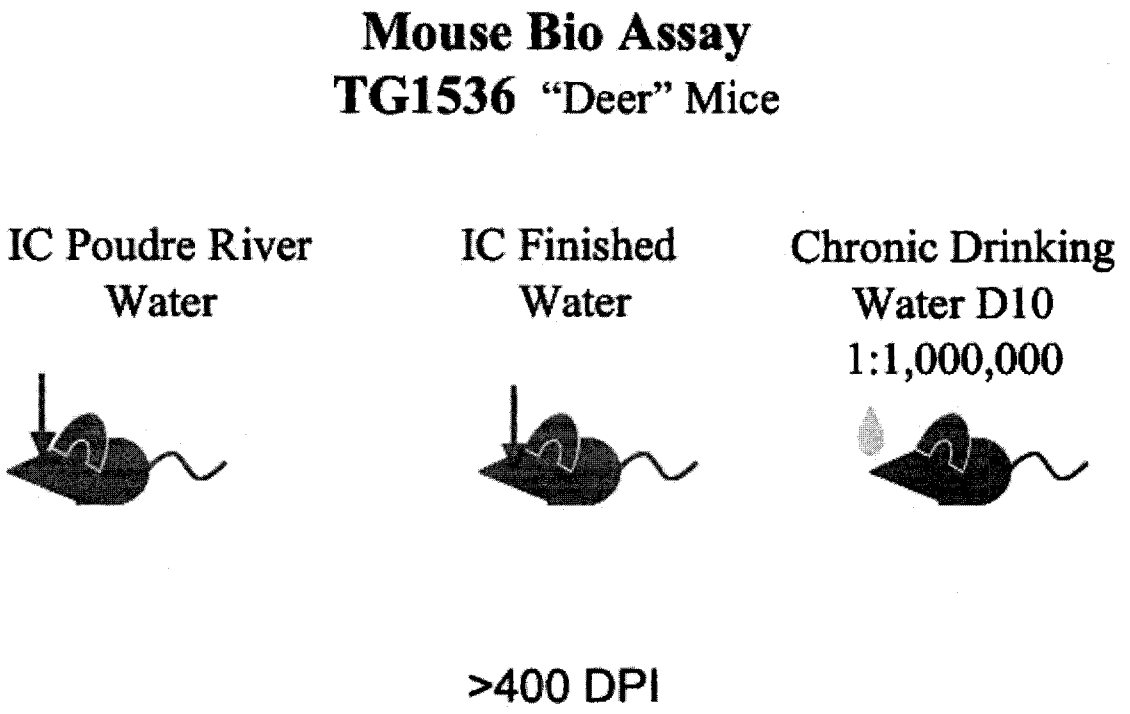


Figure 19. Mouse Bioassay Experimental Design

4. Discussion

Substantial evidence suggests that prions persist in the environment, making it a likely reservoir for CWD (29, 39, 61). Soil components such as quartz, colloids and organic carbon have been shown to bind strongly to prions. These components are washed in to surface waters during times of rain and snowmelt. Hinckley *et al.* recently reported that PrP^{res} persists in anaerobic sludge digestion in simulated waste water processing,

demonstrating its persistence through waste water processing (25).

In this study we evaluated environmental and municipal water samples from a CWD endemic area for the presence of PrP^{res}. After optimizing the sPMCA assay, we established our detection limits for CWD-positive brain dilutions in water at $1:1.32x10^7$. Recently Maluquer de Motes et. al. have demonstrated the ability of PMCA to detect PrP^{res} in sewage and seawater (34). In this study, raw water samples and drinking water treatment plant sample groups were collected and subjected to 6 rounds of sPMCA, which were replicated 3 times. PrP^{Res} was detected in two of the three replicates of Cache la Poudre River and flocculant samples from the 5-22-06 plant collection. This collection coincides with high snowmelt runoff, which washes soil and decomposing organic matter into the Poudre River. To determine the location of the PrP^{Res} within the flocculant sample, the water was centrifuged and the flocculant pellet and supernatant separated and amplified. After 6 rounds of sPMCA all detectable signal was confined to the flocculant pellet revealing that the PrP^{Res} seems to exclusively adsorb to particles in the water. Subsequent samples in the processing chain remained negative illustrating the effectiveness of the flocculation step in removing the minute amounts of PrP^{Res} . Plant samples collected at the other three time points were all negative as were environmental samples taken at different times of the year from Horsetooth Reservoir, Halligan Reservoir, Cache la Poudre River and the North Fork of the Cache la Poudre River.

Based on the D10 dilution detection limit experiment, we estimate that the 5-22-07 Poudre River and flocculant samples contained approximately 1.32 parts per billion of PrP^{Res} relative to our undiluted D10 sample. Currently there is no known infectious titre for any CWD prion strain, as there is variability in genotype susceptibility,

strain variation and individual animal titres. We therefore cannot accurately estimate the infectious dose contained therein. However, based on our *in vivo* studies in which CWD-susceptible Tg5037 mice inoculated intracerebrally with Cache la Poudre River water or chronically exposed p.o. to a 1:1,000,000 dilution of D10 spiked daily into their drinking water remain asymptomatic > 400 days after exposure, we conclude that neither group has received a lethal dose of CWD prions. This is particularly meaningful for the chronic drinking water study, as the D10 concentration is 13 times higher than that estimated in the Poudre River samples.

To address concerns regarding spontaneous PrP^{res} generation, known negative samples such as NBH controls, bottled water and Midwest water samples were evaluated for PrP^{res} signal. We detected only one positive signal in 115 of the negative controls by sPMCA, demonstrating the low rate of spontaneous PrP^{res} generation and contamination in our assay.

The catalyst for this research was to assess the risk of CWD contamination from the Colorado Division of Wildlife (CDOW) CWD research facility that lies adjacent to the City of Fort Collins water treatment plant. However, due to the fact that raw Horsetooth and Poudre River water is transported into the plant from several to scores of kilomometers removed from the CDOW, CWD contamination from this source seems logistically improbable.

Initially, it may be surprising to find any PrP^{res} in such a large volume of water, however, to understand how this could be possible, a knowledge of the Northern Colorado cervid population is needed. According to CDOW figures from 2007, there are 9,610 deer and elk in the five game units immediately surrounding the Cache la Poudre

River (74, 75) . The game units which surround the Cache la Poudre River, part of the South Platte River basin, are among the highest CWD-positive in Colorado. Symptomatic and asymptomatic positive animals can contribute to the environmental CWD load via bodily fluids such as saliva, blood, urine and feces, shedding several times their body weight in possibly infected excreta over the course of their lifetime, as well as through decomposing carcasses from infected animals. Murayama et.al. found that PrP^{res} was shed intermittently in the urine of infected mice (40). More recently, Gonzalez-Romero *et al.* were able to detect PrP^{res} in 80% of the urine from experimentally infected hamsters by PMCA. Although the concentration of PrP^{res} was approximately 10-fold lower than blood, it remained infectious upon excretion. The authors concluded that urine is a possible source of transmission (20). Feces from infected hamsters has also been shown to contain PrP^{res}, strongly suggesting that feces is also a source of environmental contamination (57).

Because the Cache la Poudre River is a source of human drinking water, there is concern regarding the potential risk to humans. However, our *in vivo* infection studies in which positive water samples were injected IC into mice show that at >365 dpi all animals are asymptomatic. Because these animals are transgenic for cervid PrP, they are highly susceptible to CWD. The lack of illness suggests that the dose of CWD present in the environmental and plant samples is not high enough to cause disease, nor is a chronic daily exposure to $1:1x10^6$ of infected brain via drinking water. This dose is approximately 13 times greater than that estimated from our detection limit data. Furthermore, current data indicates the presence of a significant species barrier between CWD and humans (31) and no increase in the incidence of human prion diseases has

been detected in Colorado, despite the consumption of deer and elk meat from areas that are know to have significant amounts of CWD (4, 31, 37). Meat consumption would give a substantially higher dose than that which was detected in the raw Poudre River water. Taken together, these data strongly suggest that the likelihood of the minute amount of PrP^{res} detected is highly unlikely to cause disease in humans or animals. However, they do not eliminate environmental contamination as a source of CWD transmission. Infected excreta and carcasses may sufficiently concentrate prions in local environments where deer and elk congregate, such as dens or wallows, to horizontally transmit CWD.

Removal of PrP^{res} from liquids such as bodily fluids has become important due to concerns about iatrogenic infection of new variant Creutzfeldt-Jakob disease, associated with exposure to BSE- infected beef, from blood transfusions. Several attempts have been made to remove PrP^{res} by filtration and have met with some success, although the PMCA technique was not used to detect minute amounts of PrP^{res} (72, 81). It is important to note that Yunoki et al. sonicated their samples prior to filtering to mimic current medical protocols. This step would break apart fibrils into much smaller fragments as in PMCA, making them harder to remove via filtration. Although conventional filtering has not been particularly successful at removing PrP^{res} from aqueous mediums, our data indicates that ultrafilration using a 20nm filter significantly reduced a relatively high CWD spike in water (130x our detection limit) after 6 rounds of sPMCA (6, 72, 81). Although it is doubtful that the PrP^{res} detected is of concern to human health, ultrafiltration of municipal water is currently possible with 20nm ultrafiltration systems. There are several in place in the United States, including Thornton, Colorado.

These data show for the first time that PrP^{res} can be detected in environmental water samples from a CWD-endemic area with sPMCA, however at minute concentrations that do not appear to cause illness in susceptible hosts. Additionally, we have demonstrated that PrP^{res} contamination positively correlates with increased runoff from snowmelt and can be eliminated with PK pre-treatment and greatly decreased with a 20 nm filter. This work shows that sPMCA can be utilized to evaluate environmental water samples for PrP^{res}.

Chapter 3

PROTEIN MISFOLDING CYCLIC AMPLIFICATION (PMCA) AND PrP^{Res} DETECTION IN SOIL

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are an unusual group of infectious diseases that lead to progressive neurodegeneration and death. Included in this group are bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer, elk and moose and kuru and Creutzfeldt-Jakob disease in humans (23). Considerable evidence supports the hypothesis that the protease-resistant misfolded prion protein (PrP^{Res}) is the causative agent in TSEs (7, 19, 23, 30, 45). Exposure of PrP^{C} to β-sheet rich conformer results in conversion, via a yet unidentified process, into more PrP^{Res}, which is characteristically resistant to proteinase- K (PK) digestion (45). PrP^{Res} can be detected in wide variety of tissues such as tonsils, spleen, retina, skeletal muscle, peripheral nervous system, endocrine organs, blood, saliva, lymph nodes, spinal cord and most strongly in the brain (2, 26, 36, 67, 78).

Chronic wasting disease (CWD) is endemic to Northeastern Colorado and was first described in mule deer in 1967 in Fort Collins (79). The area surrounding Fort Collins has the highest incidence of CWD in Colorado (76), but positive deer (*Odocoileus hemionus and virginianus*) and elk (*Cervus elaphus nelsoni*) have been found throughout Colorado, Wyoming, Montana, Minnesota, Wisconsin, Oklahoma, New York, Kansas,

Nebraska, South Dakota and parts of Canada (78).

CWD transmission is quite efficient, as the incidence of CWD in some wild populations has been documented to be as high as 30% (78). Unlike BSE, CWD has been shown to efficiently transmit horizontally, (59), the exact mechanism of which remains the focus of numerous studies (36, 39). Indirect exposure via the environment may be a significant factor in transmission as saliva and blood have proven to be infectious, and current work suggests that urine and feces may also be a route of transmission (36, 39). Research indicates that prions are able to remain infectious in the environment and transmit disease years after contamination (9, 29, 52, 70). Both mineral and organic soil components, have been shown to strongly adsorb prion proteins (29, 48, 52, 61). Furthermore, soil adsorption of hamster scrapie has been shown to enhance the oral transmission of the disease by increasing the infectious titer by a factor of 680 when compared to the unbound hamster scrapie (28). In nature, decomposition of CWD-positive carcasses, bodily fluids such as saliva and urine and feces all contact soil, which can be consumed during foraging and drinking (2, 26, 34-36, 39, 40, 67). The ability to detect PrP^{Res} on soil in the environment would further our understanding of the extent of environmental contamination of natural and agricultural areas as well as evaluate sites for future agrarian use.

The detection of small amounts of PrP^{res} is quite limited due to the low of sensitivity of commonly used detection methods such as **Western Blotting** and immunohistochemistry. This lack of sensitivity, therefore, prohibits the detection of small amounts of infectious prions in animal tissues and environmental samples by these methods. Protein misfolding cyclic amplification (PMCA) is an *in vitro* technique that

enables the amplification of minute amounts of PrP^{res} by allowing the rapid conversion of PrP^{c} to PrP^{res} (12, 55, 56, 66). Initial PMCA experiments were conducted utilizing hamster-adapted scrapie strain 263 K (56). Brain homogenate is generated from an uninfected animal and is used as the source of PrP^{c} substrate. The unknown sample is then added, which if positive, will begin to convert the PrP^{c} to PrP^{res}, increasing its concentration to a level that can be detected by conventional western blotting. As mentioned previously, infectious prion proteins aggregate into fibrils that appear to slow conversion of PrP^{c} to PrP^{res} (18). To successfully generate PrP^{res} , these aggregates must be disrupted to create smaller "seeds" which can then continue to convert PrP^{c}. In PMCA this is accomplished by the use of a sonication step that disrupts prion aggregates (fig. 4). To determine if the newly formed PrP^{res} possessed the ability to continue to convert more PrP^{C} to PrP^{res}, serial PMCA (sPMCA) was developed (5). This modification, which implements the placement of sample into fresh brain homogenate after a specific number of PMCA cycles, increased amplification with each sPMCA round (5). Castilla et.al. have shown that sPMCA gives a 6,600-fold increase in sensitivity when compared to standard detection methods and is able to able to detect as few as 8,000 molecules of scrapie PrP^{res} (12). sPMCA has been used to successfully amplify BSE from cows, scrapie from mice, goats and sheep, CJD and variant CJD from humans and CWD from deer and elk (32, 66).

In this study we assessed the ability of sPMCA to detect minute amounts of CWD in both laboratory inoculated soil samples and environmental soil samples. Although sPMCA has shown to be useful in detection of in various water samples, we were unable to detect PrP^{res} in CWD spiked soil or soil from a ranch which houses CWD-positive

animals. Modifications of our current technique will be required before sPMCA can be used to assess soil samples.

2. Methods and Materials

2.1 Mice

Tg(cerPrP)5037 mice were generated in the Telling laboratory in a similar fashion to Tg1536 (10), replacing the SHa cosmid vector with the MoPrPXho expression vector and resulting in a wider tissue distribution of CerPrP expression that was increased to 6 times the normal expression of MoPrP in wild type mice. Mice were housed and maintained at Lab Animal Resources, in accordance with protocols approved by the Institutional Animal Care and Use Committee at Colorado State University.

2.2 Preparation of brain homogenates

Tg1536 or Tg5037 mice were perfused with 30 ml of 1x phosphate buffered saline (PBS) plus 5 mM EDTA prior to removal of the brain. A 10% normal brain homogenate (NBH) (wt/vol) was prepared in PMCA conversion buffer (12). Briefly, brains were halved and snap frozen in liquid NO_2 then weighed. The halves were **weighed** and the appropriate volume of PMCA buffer 1 containing 1X PBS, 150 mM NaCl, 4 mM EDTA plus the complete Protease Inhibitor Cocktail (Roche) was added to tubes with glass beads for FastPrep homogenizing (Biogene) to generate a 10% wt/vol solution. An equal volume of PMCA buffer 2 (PMCA 1 buffer without protease inhibitor plus 2.0% triton X-100) was added and samples were left to incubate on ice for 20 min then clarified by centrifugation at 1000 RPM for 5 minutes. The supernatant was carefully removed and

frozen at -80° C.

2.3 sPMCA procedure

The sPMCA (serial protein misfolding cyclic amplification) technique used was based on that described by Saa et al, however, a number of modifications were made (55). To establish a water detection limit and evaluate environmental samples, 25µl of each sample was added to individual 200 µl PCR sample tubes sealed shut with parafilm™ preventing cross contamination containing 25µl of NBH. The tubes were then subjected to 40 sec pulses of sonication at 37° C, power setting 7, every 30 min for a 24 hr period using a 3000Mp sonicator (Misonix). After 24 hrs, 25µl of each sample was mixed, removed and added to 25µl of fresh NBH. Samples were again sonicated as described above constituting 1 round of PMCA. This process was repeated six times.

2.4 CWD-spiked soil detection limit

10mg of CWD negative soil was added to 90µl of deionized H2O to make a 10% mud. The mud was then spiked with a 10% solution of CWD-positive brain made from a deer (D10) that was terminally sick with CWD. 2-fold dilutions were made spanning 1:1600 through 1:816,200, then amplified by sPMCA for 6 rounds.

2.5 Environmental soil samples

Soil samples were collected from Fort Collins, Colorado, Adrian, Michigan and from pens that have contained CWD positive elk near Fort Collins, Colorado. Soil samples were sifted to remove larger rocks and plant material then weighed. 90µl of deionized H2O was added to 10mg of sample soil to make a 10% mud.

2.6 PK digestion and Western blot

Samples were treated with 50 μg/ml of proteinase-K (Roche) for 30 min at 47° C.Amplified samples were fractionated using 12% Bis-Tris SDS-PAGE gels (Invitrogen), transferred to Immobilon PVDF membrane (Invitrogen) and probed with 1:20,000 Bar 224/HRP-conjugated anti-PrP monoclonal antibody (SPI bio) in Superblock (Pierce). Western blots were visualized using the FujiDoc gel documentation system (Fuji).

3. Results

To ascertain our ability to detect D10 CWD-positive brain spikes in soil, 1:2 D10 dilutions were made then added to negative soil and amplified for 6 sPMCA rounds. We were unable to detect even the lowest D10 spikes (fig. 20. Soil samples collected from a low cervid use area in Colorado and soil from states not currently known to have CWD were all negative for PrP^{res} after 6 rounds of sPMCA (fig. 21) . In an attempt to detect PrP^{res} in an environmental soil and water sample, soil from a heavily used wallow on an elk ranch housing CWD-positive elk was evaluated. Both the water and soil from the wallow were negative for PrP^{res} (fig. 22).

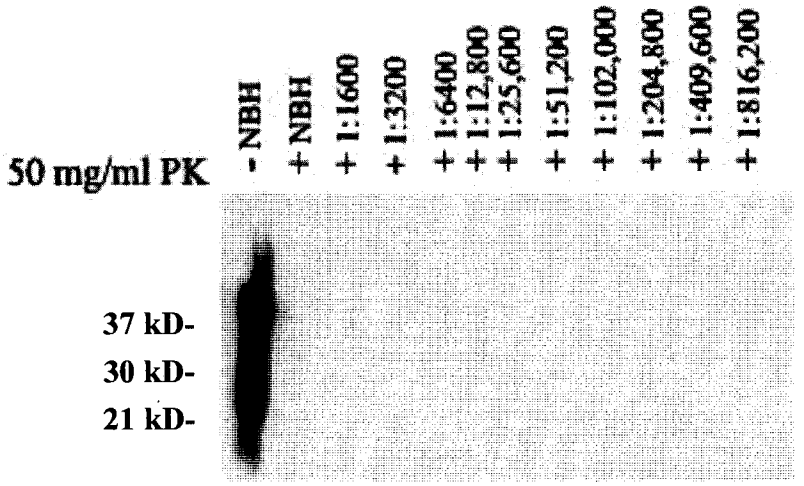


Figure 20. sPMCA on 2-fold D10 dilutions 1:1600 – 1:816,200 D10 in soil.
NBH- Normal brain homogenate negative controls. We were unable to detect the D10 spikes in any of the samples after 6 rounds of sPMCA.

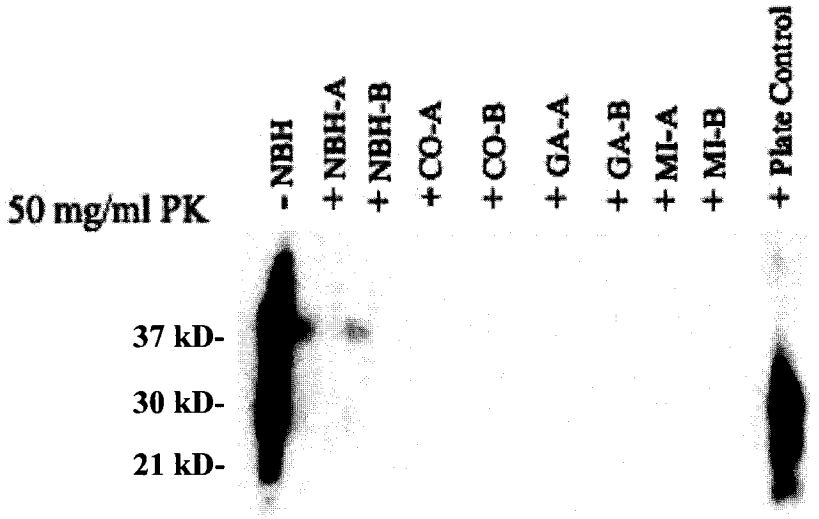


Figure 21. sPMCA on Soil from Colorado, Georgia and Michgan.
NBH- Normal brain homogenate negative controls. All samples were negative for PrP^{Res} after 6 rounds of sPMCA.

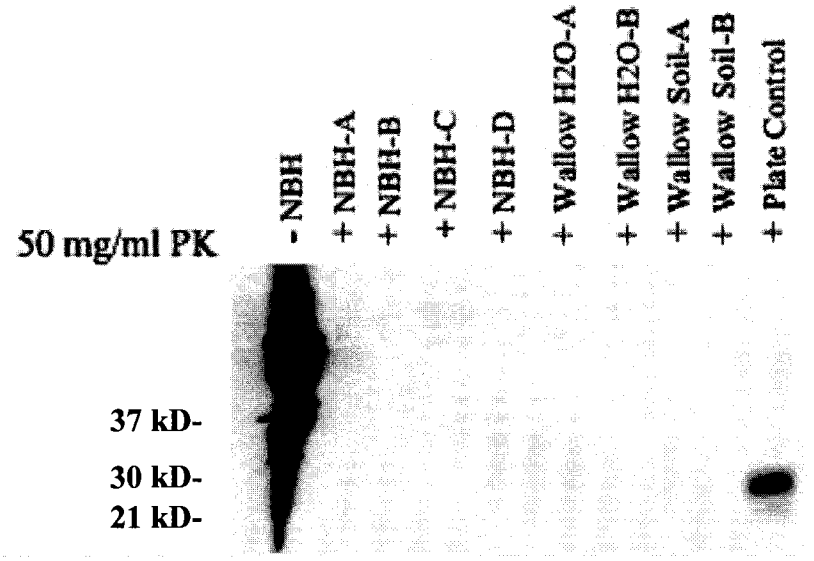


Figure 22. sPMCA of water and soil from a wallow on a CWD-positive ranch.
NBH- Normal brain homogenate negative controls. All samples were negative for PrP^{Res} after 6 rounds of sPMCA.

4. Discussion

sPMCA has proven to be a useful tool in the detection of PrP^{Res} in Cache la Poudre River and flocculation water samples, however, we were unable to detect PrP^{Res} in any of our D10 CWD spikes even at levels that would have been easily detected in our water samples. As discussed in the literature (61), PrP^{res} adsorbs tightly to soil particles, making them difficult to extract. Our data suggests that CWD is very tightly adsorbed to the soil particles in our samples, requiring us to optimize our sPMCA protocol. Several strategies have been implemented by other researchers such as treatment with SDS (61), sarkosyl (15) and electroelution (52). Incubation with SDS seems like the most straightforward approach and should be tried next. Once optimized, sPMCA could be a useful tool to evaluate the soil of land being considered for wild game ranching or other agricultural uses, as well as contributing more information on the transmission of CWD in the wild.

Chapter 4

CONCLUSION

Serial protein cyclic misfolding amplification (sPMCA) has proven to be effective in amplifying PrP^{Res} material from a variety of animal tissues and more recently it has been used to detect PrP^{res} spiked into sea and sewage water. In this study we found our detection limit of CWD material in deionized water to be 1:1.33 $x10^7$ and for the first time sPMCA was used to detect PrP^{Res} in surface and processed water from a CWD endemic area. Samples were collected at four different times of the year and were amplified for 6, 24 hr rounds of sPMCA. Samples containing PrP^{Res} positively correlated with a time of high snowmelt runoff, during which soils and organic carbon material were flushed into surface waters. To determine whether PrP^{Res} was adsorbed to particulate matter in the water, positive flocculant water was centrifuged and the flocculant pellet was separated from the supernatant water. Both were amplified by sPMCA. PrP^{Res} was detected exclusively in the flocculant pellet illustrating that it is not floating free within the water samples, but rather adsorbed to particulate matter. The flocculation step in water processing appears to effectively remove any detectable PrP^{Res}, as subsequent stages of processing were negative.

Analysis of normal brain homogenate controls revealed that the rate of false positives due to contamination or spontaneous conversion of PrP^{C} into PrP^{Res} was extremely low in

this study, which had a specificity of 99.2% . To further validate the nature of the positive PrP^{Res} signals detected in the water samples positive Cache la Poudre River water was pre-treated with PK prior to sPMCA. PK pre-treament abrogated the signal in the sample, illustrating that the PrP^{Res} was present prior to sPMCA.

Environmental transmission of CWD has been documented in captive cervids. The exact method of transmission has yet to be elucidated, however, the results of this study indicate that although water from the Cache la Poudre River contains minute amounts of PrP^{Res} at times of high snowmelt, it is not enough to cause disease in our cervidized transgenic mice, minimizing its probable contribution to CWD infection in the wild. However, smaller bodies of surface water, such as streams, seeps and vernal pools, could be a route of oral transmission and likely contain a much higher concentration of PrP^{Res}. Further research is needed to determine the role of these water sources in the transmission of CWD in the wild. sPMCA seems to be a useful tool in gaining this information.

Unlike our success in detecting PrP^{Res} in water, our current sPMCA protocols were ineffective at detecting CWD-positive spikes in soil. A low dilution that can be readily detected in water was negative in the soil samples. This suggests that adsorption to soil particles is very strong and further optimization is required to be able to detect PrP^{Res} in soil using sPMCA and use to evaluate sites for CWD contamination. Work done in other laboratories suggests that detergents, SDS and sarkosyl and electroelution can dissociate prions from soil (15, 52, 61). A simple strategy to employ first would be to incubate soil samples with SDS at varying concentrations and temperatures. Further work on the role of soil and transmission will be evaluated by another graduate student within our laboratory.

To evaluate the role of water in CWD transmission it will be necessary to sample from smaller bodies of water within the endemic area perform sPMCA and conduct bioassays. Although the Cache la Poudre River water did not contain enough PrP^{res} to induce illness in our bioassay, smaller slower moving waters may contain enough material to cause illness. One exposure may not be sufficient to cause disease but repeated exposures may. I intend to conduct this work in my postdoctoral position.

APPENDICES

A. Sample Replicates 2 and 3 from chapter 2 - Tube Method

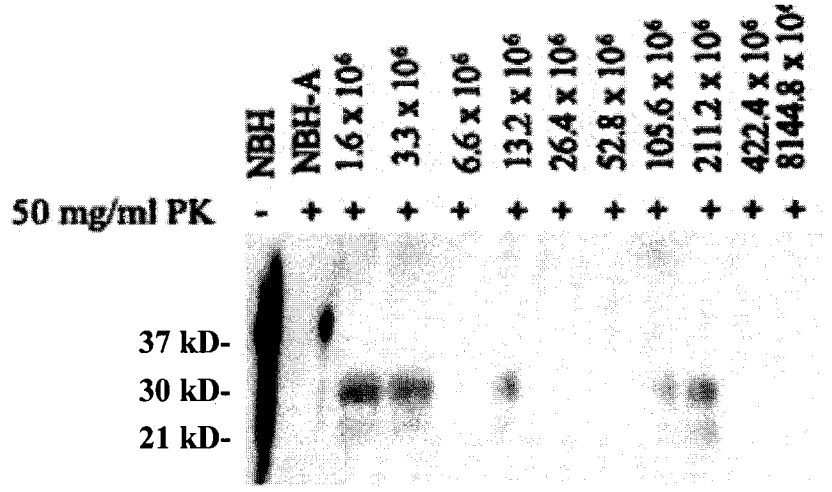


Fig. 23. sPMCA on 2-fold D10 dilutions 1:1600 – 1:422.4 x10^6 Rep 2. NBH-Normal brain homogenate negative controls. Greatest detectable D10 concentration after 6 sPMCA rounds was 1:13.2 x 10^6 . Signals were present in 105.6 and 211.2 x 10^6, however, these were only present on one of the 3 gels, . Therefore, they do not meet our critieria to be considered positive.

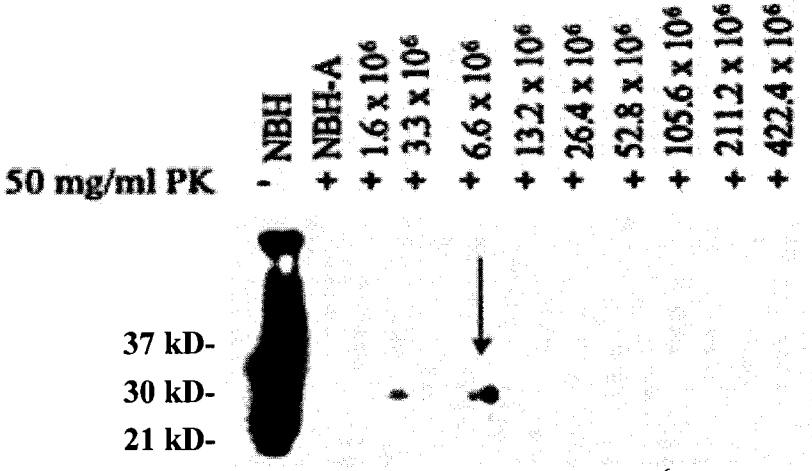


Fig. 24. sPMCA on 2-fold D10 dilutions 1:1600 – 1:422.4 x10^6 Rep 3. NBH-Normal brain homogenate negative controls. Greatest detectable D10 concentration after 6 sPMCA rounds was 1:6.6 x 10^6 .

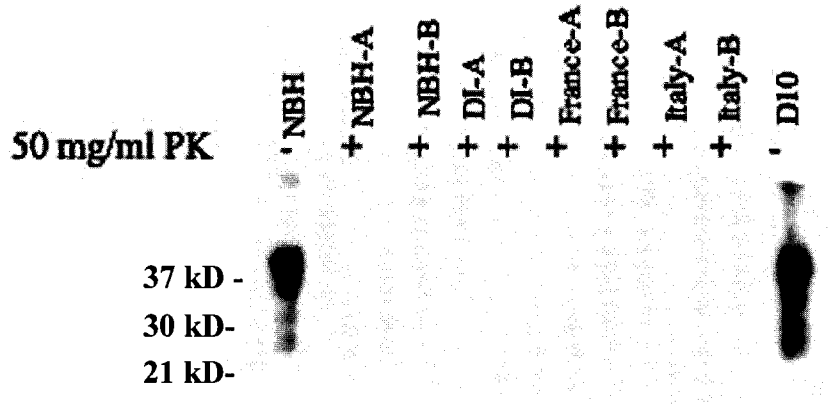


Fig 25. sPMCA of bottled water Rep 2.
D10- Unamplified 10% infected brain positive controls. NBH- Normal brain homogenate negative controls. D10+ -1:100,000 amplified positive amplification control. DI- Electrolyte-enhanced deionized water. FR- Natural spring water bottled in France. IT- Italian sparkling mineral water bottled in Italy. All bottled water sample replicates were negative.

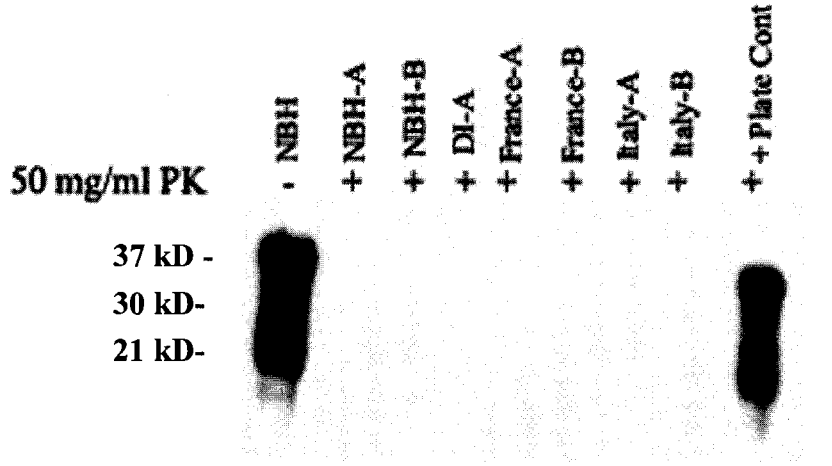


Fig 26. sPMCA of bottled water Rep 3.
D10- Unamplified 10% infected brain positive controls. NBH- Normal brain homogenate negative controls. D10+ -1:100,000 amplified positive amplification control. DI- Electrolyte-enhanced deionized water. FR- Natural spring water bottled in France. IT- Italian sparkling mineral water bottled in Italy. All bottled water sample replicates were negative.

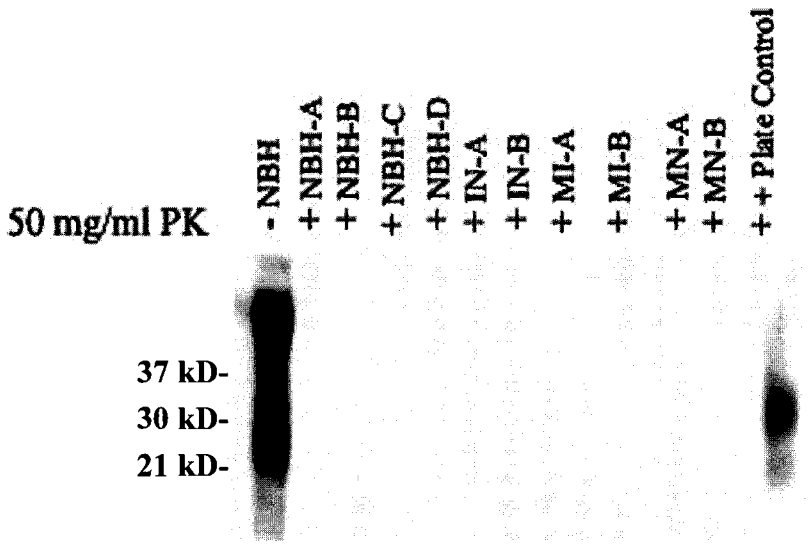


Fig 27. sPMCA of municipal water samples from the Midwestern United States Rep 2.
D10- Unamplified10% infected brain positive controls. NBH- Normal brain homogenate negative controls. Plate Control+ -1:100,000 amplified positive brain. IN- Indiana. MI- Michigan. MN- Minnesota. All water sample replicates were negative after 6 sPMCA rounds.

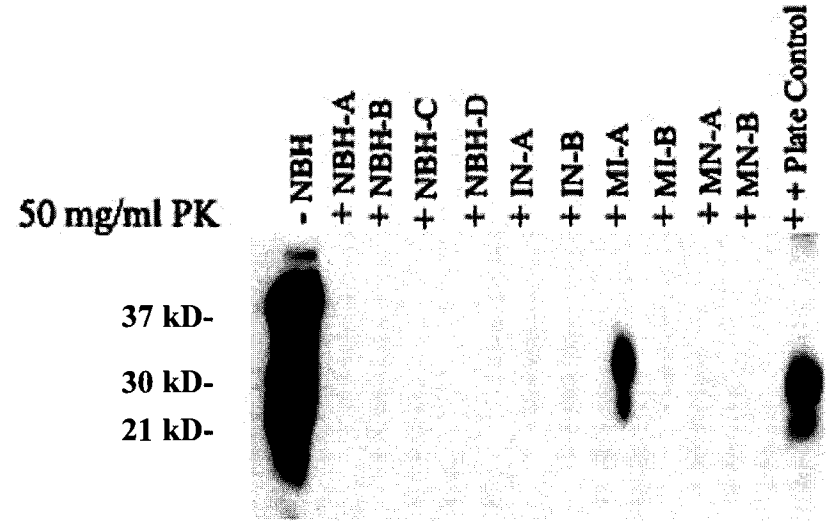


Fig 28. sPMCA of municipal water samples from the Midwestern United States Rep 3.
D10- Unamplified10% infected brain positive controls. NBH- Normal brain homogenate negative controls. +Plate Control -1:100,000 amplified positive brain. IN- Indiana. MI- Michigan. MN- Minnesota. Although there is a spot in the Michigan A sample, it does not line up with the bands of the positive plate control.

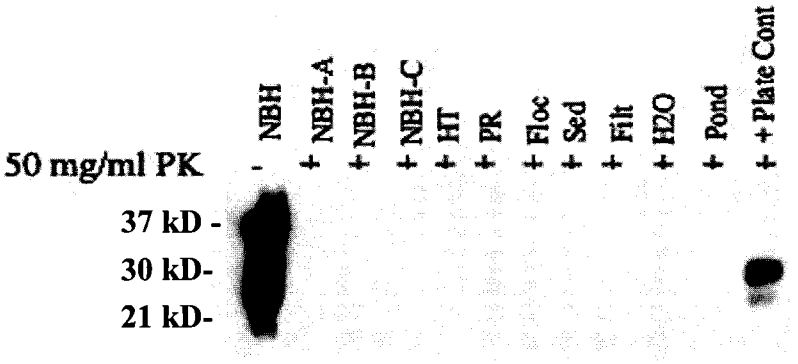


Fig. 29. 3-29-07 Plant sample Rep 2.
NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. + sPMCA control- 1:100,000 amplified brain. All samples were negative for PrP^{res} after 6 sPMCA rounds..

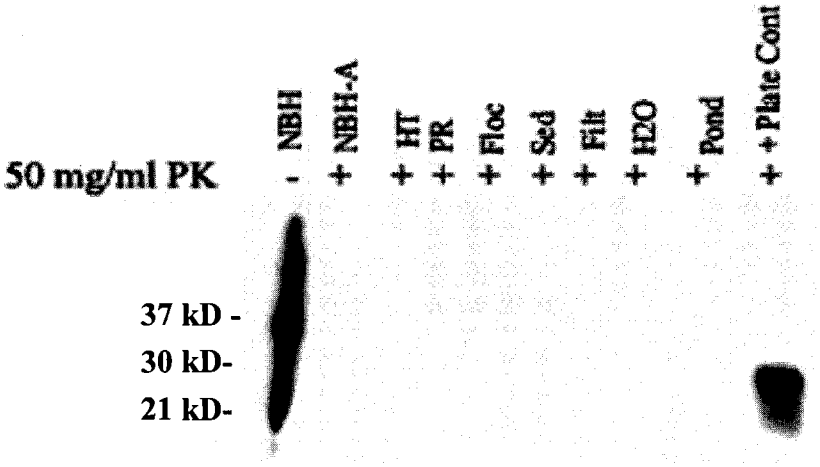


Fig. 30. 3-29-07 Plant sample Rep 3.
NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. + sPMCA control 1:100,000 amplified positive brain. After 6 sPMCA rounds all samples were negative for PrP^{res} .

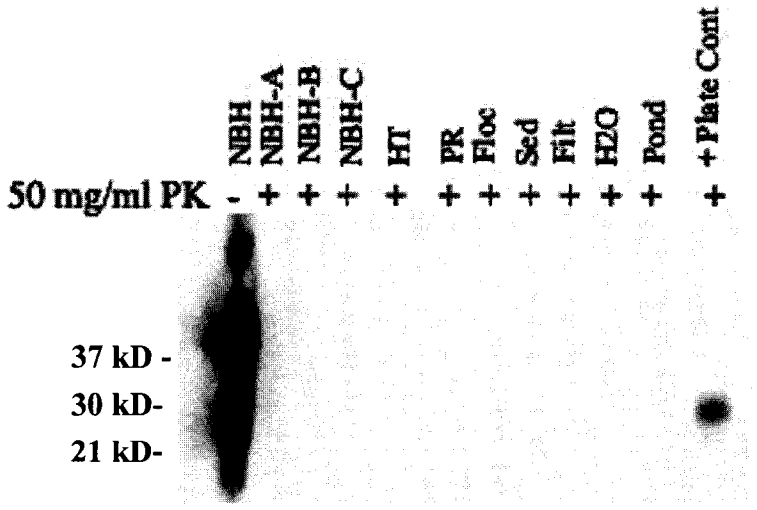


Fig. 31. 5-10-07 Plant sample Rep 2.
NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control-1:100,000 amplified positive brain. All samples were negative for PrP^{res} after 6 rounds of sPMCA.

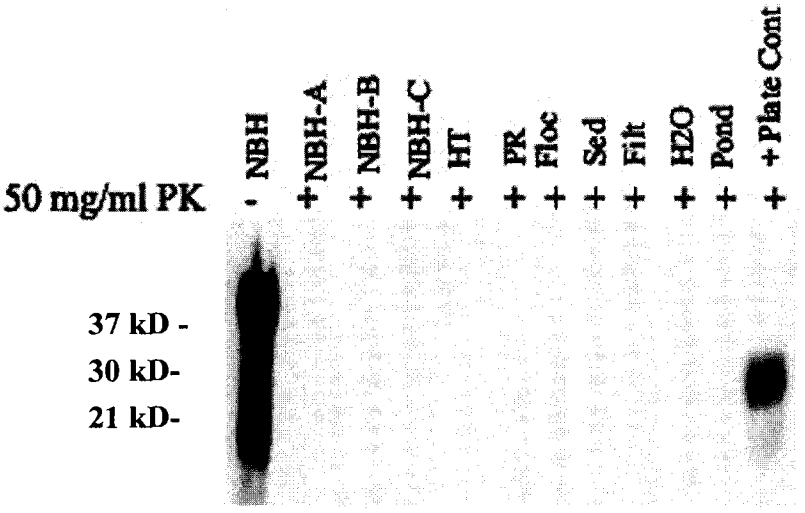


Fig. 32. 5-10-07 Plant sample Rep 3.
NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control-1:100,000 amplified positive brain. All samples were negative for PrP^{res} after 6 rounds of sPMCA.

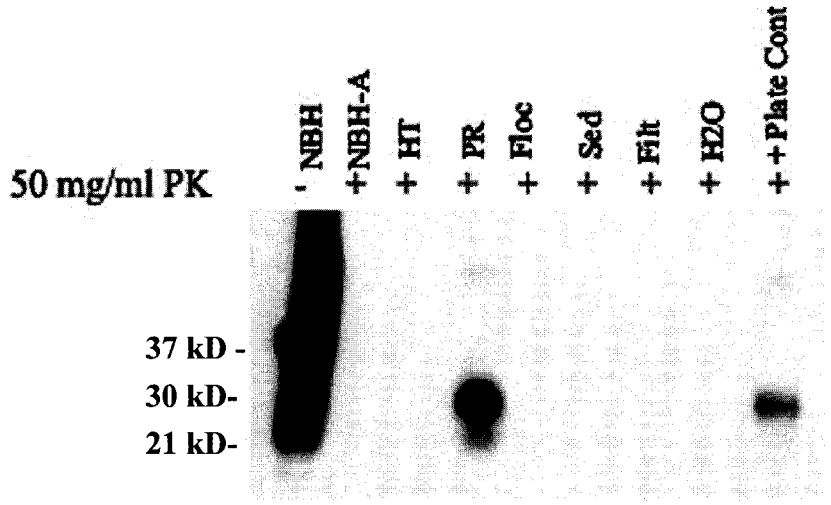


Fig. 33. 5-22-07 Plant sample Rep 2.
NBH- Normal brain homogenate negative controls. **A**. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control-1:100,000 positive brain control. After 6 sPMCA rounds, PrP^{res} was detected in the Cache la Poudre River sample in this replicate.

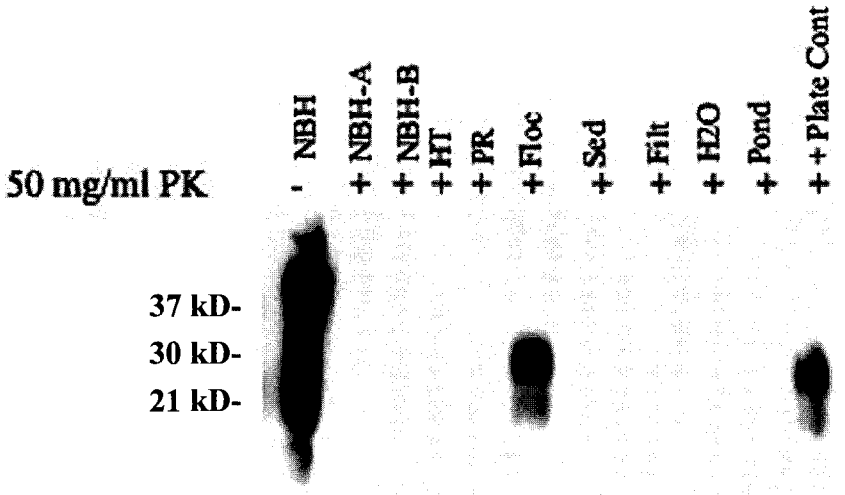


Fig. 34. 5-22-07 Plant sample Rep 3.
NBH- Normal brain homogenate negative controls. **A**. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. PR-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filt-Water post filtering. H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control-1:100,000 amplified positive brain. After 6 sPMCA rounds, PrP^{res} was detected in floc.sample in this replicate.

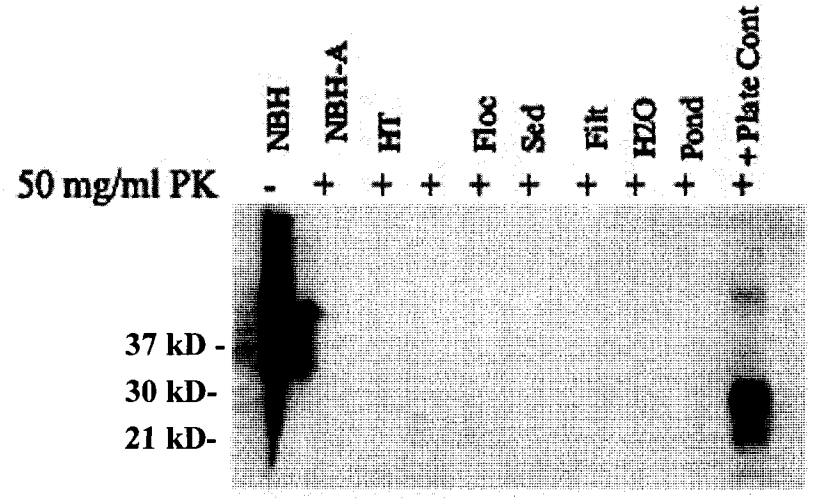


Fig. 35. 7-23-07 Plant sample Rep 2.
NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. Finished H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control-1:100,000 amplified positive brain. All samples were negative for PrP^{res} after 6 sPMCA rounds.

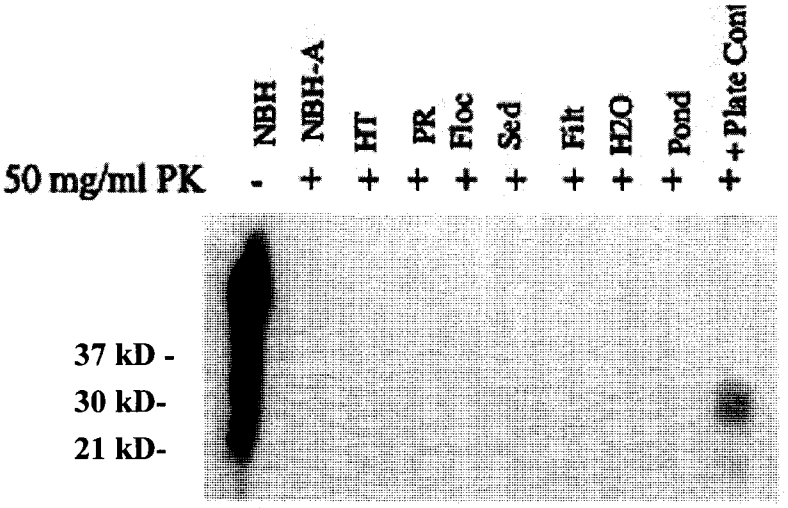


Fig. 36. 7-23-07 Plant sample Rep 3.
NBH- Normal brain homogenate negative controls. Water samples from City of Fort Collins drinking treatment plant: HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. Finished H2O-Water ready for distribution. Pond-Water from filter backwash pond. + sPMCA control- 1:100,000 amplified positive brain. All samples were negative for PrP^{res} after 6 sPMCA rounds.

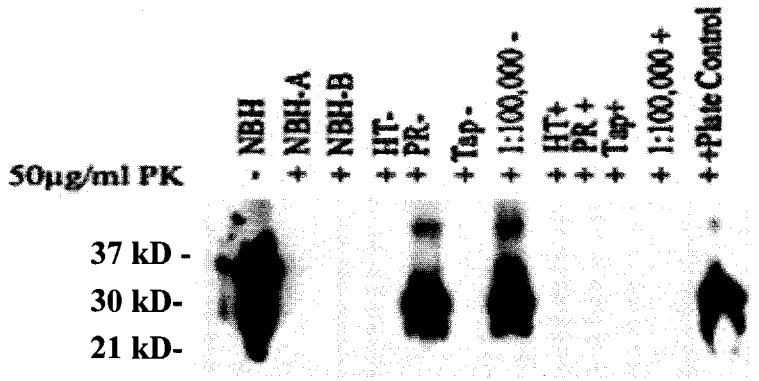
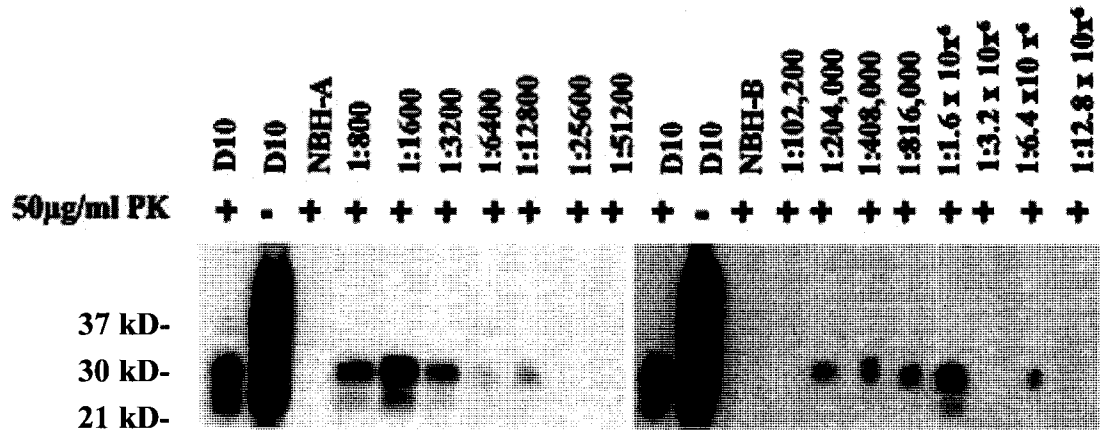


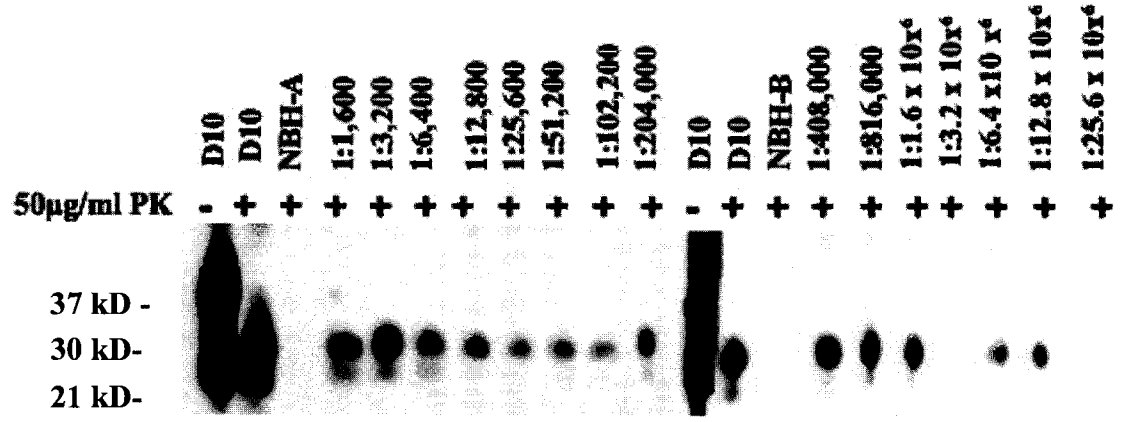
Figure 37. Ultrafiltration of PrP^{res} in water samples.
NBH- Normal brain homogenate negative controls. HT- -Horsetooth reservoir water unfiltered. HT+ - Horsetooth reservoir filtered with a 20 nm syringe filter. P.River- -Cache la Poudre River water unfiltered. P.River + -Cache la Poudre River water filtered with a 20 nm syringe filter. 1:100,000- - D10 dilution unfiltered. 1:100,000+ - D10 dilution filtered with a 20 nm syringe filter. + Control – D10 1:100,000 amplified positive plate control. Filter removed signal in PR and 100,000 after 6 sPMCA rounds.

B. sPMCA 96-Well Plate Assay Data

The original Misonix sonicator was fitted to hold a 96 well PCR plate. The water and soil experiments were initially conducted using a 96 well plate. The resulting data showed positive signal in most of the water samples tested. We found this surprising, but replicates repeatedly produced the same results. After acquiring a new sonicator, which was accompanied by an acrylic insert that held individual 200 μl PCR tubes, we discovered that there was sample migration on the plates. This necessitated repeating all of the water and soil experiments in individual tubes. The following data is from the plate experiments. The disparity between these data and the tube data illustrates the necessity of using the individual tube method rather than the plate method.



A.



B.

Fig. 38. sPMCA on 2-fold D10 dilutions 1:1600 – 1:25.6 x10^6.
NBH-Normal brain homogenate negative controls. D10- Unamplified10% infected brain positive controls.
A..The greatest detectable D10 dilution detected after 4, 24hr rounds of sPMCA was 1:1.6 x10^6.
B..The greatest detectable D10 dilution detected after 7, 24hr rounds of sPMCA was 1:12.8 x10^6.

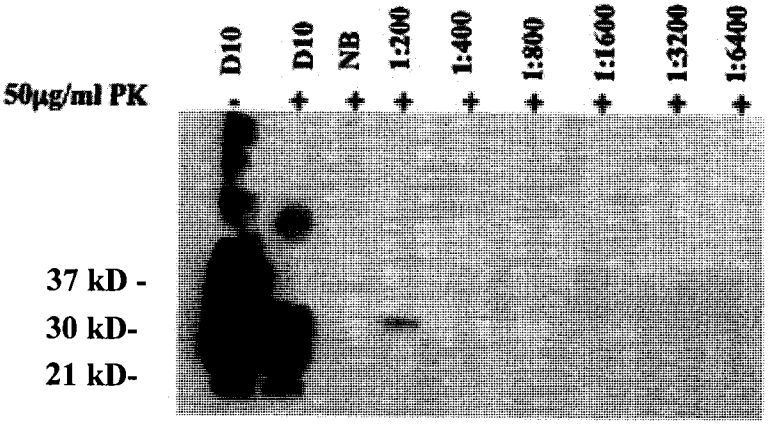


Fig. 39. Unamplified 2-fold D10 dilutions 1:200 - 1:6400.
NBH-Normal brain homogenate negative control. D10- Unamplified 10% infected brain positive controls. The greatest detectable D10 dilution detected without the use of sPMCA was 1:200.

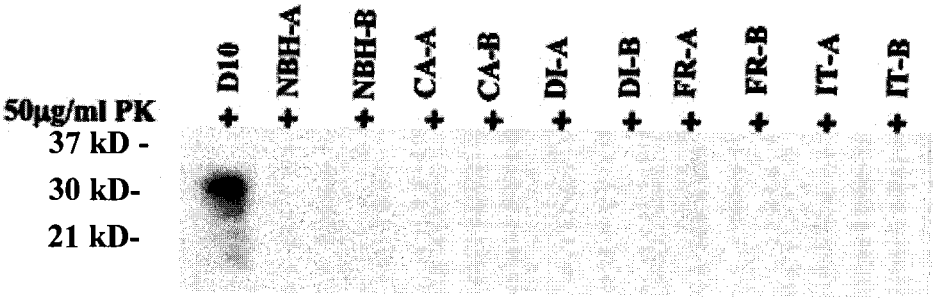


Fig 40. sPMCA of bottled water.
D10- Unamplified 10% infected brain positive controls. NBH- Normal brain homogenate negative controls. D10+ -1:100,000 amplified positive amplification control. DI- Electrolyte-enhanced deionized water. FR- Natural spring water bottled in France. IT- Italian sparkling mineral water bottled in Italy. Figure represents one of three, 4 sPMCA round replicates executed. Each replicate was done in duplicate (A or B). All bottled water sample replicates were negative.

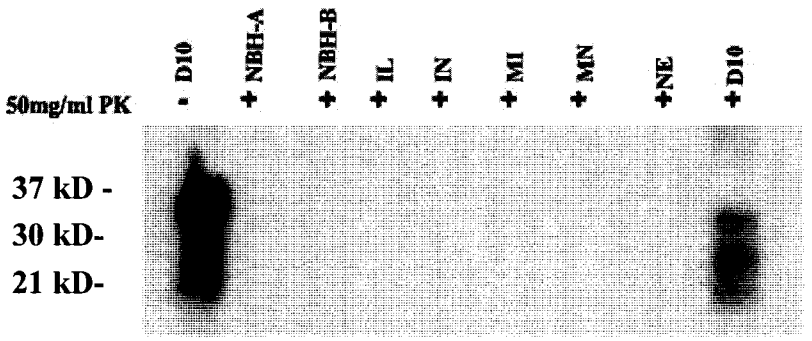


Fig 41. sPMCA of municipal water samples from the Midwestern United States.
D10- Unamplified10% infected brain positive controls. NBH- Normal brain homogenate negative controls. D10+ -1:100,000 amplified positive amplification control. IN- Indiana. MI- Michigan. MN- Minnesota.. Figure represents one of three, 4 sPMCA round replicates executed. Each replicate was done in duplicate (A or B). All water sample replicates were negative after 5 sPMCA rounds.

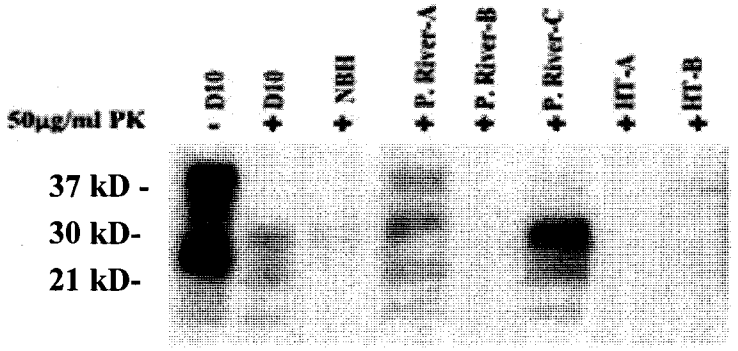


Fig. 42. 9-27-06 Raw water samples collection 1.
NBH- Normal brain homogenate negative controls. D10- Unamplified 10% infected brain positive controls. P.River-Cache la Poudre River water. HT-Horsetooth reservoir water. Incoming raw source water collected in City of Fort Collins drinking treatment plant. Each sample was done in duplicate HT (A, B) or triplicate PR (A, B & C). Results showed that PrP^{res} was detected in 2 of the 3 PR samples and no signal was detected in HT samples after 5 sPMCA rounds.

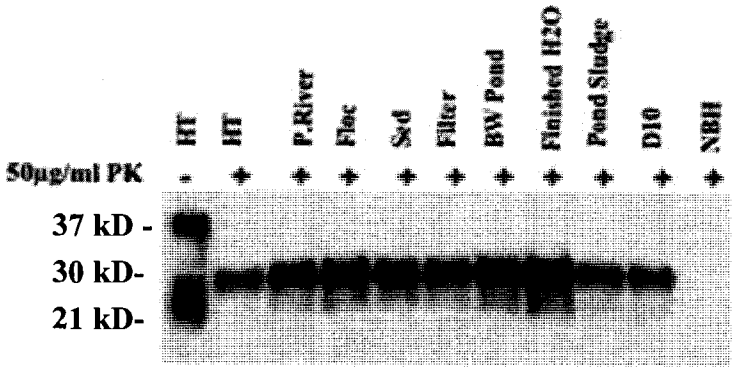


Fig. 43. 3-29-07 Plant sample collection 1.
D10- Unamplified 10% infected brain positive controls. NBH- Normal brain homogenate negative controls. HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. BW Pond-Water from filter backwash pond. Finished H2O-Water ready for distribution. Pond Sludge-Sludge from bottom of filter backwash pond. Water samples from City of Fort Collins drinking treatment plant. This western blot is representative of 5 separate replicates after 5 sPMCA rounds, 3 of which were done in duplicate. Western blotting showed that PrP^{res} was detected in the majority of the water samples taken on this date.

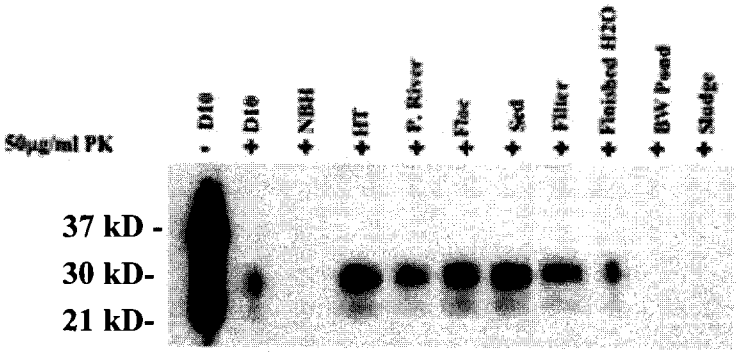


Fig. 44. 5-9-07 Plant sample collection 2.
NBH- Normal brain homogenate negative controls. D10- Unamplified 10% infected brain positive controls. HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. BW Pond-Water from filter backwash pond. Finished H2O-Water ready for distribution. Pond Sludge-Sludge from bottom of filter backwash pond. Water samples from City of Fort Collins drinking treatment plant. This western blot is representative of 3 separate replicates after 5 sPMCA rounds, each were done in duplicate. Western blotting showed that PrP^{res} was detected all samples at least once with the exception of post sediment water and filter backwash pond sludge.

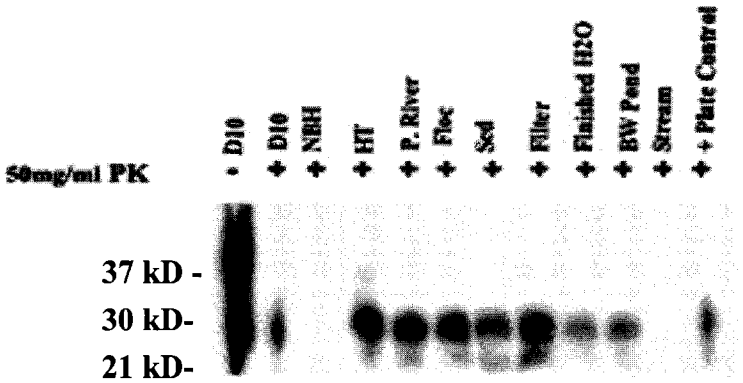


Fig. 45. 5-22-07 Plant sample collection 3.
NBH- Normal brain homogenate negative controls. D10- Unamplified 10% infected brain positive controls. HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. BW Pond-Water from filter backwash pond. Finished H2O-Water ready for distribution. Water samples from City of Fort Collins drinking treatment plant. This western blot is representative of 3 separate replicates after 5 sPMCA rounds, each were done in duplicate. Western blotting showed that PrP^{res} was detected all samples at least twice.

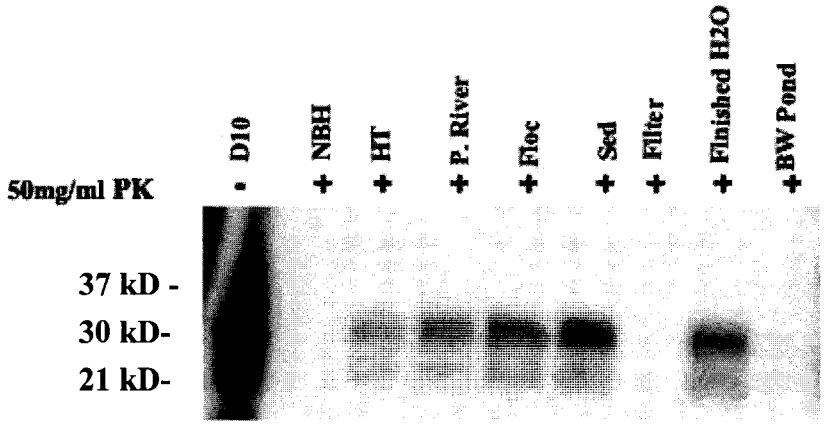


Fig. 46. 7-23-07 Plant sample collection 4.
NBH- Normal brain homogenate negative controls. D10- Unamplified 10% infected deer brain positive controls. HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Floc-Post flocculation water. Sed-Post sedimented water. Filter-Water post filtering. Finished H2O-Water ready for distribution. BW Pond-Water from filter backwash pond. Water samples from City of Fort Collins drinking treatment plant. This western blot is representative of 3 separate replicates after 5 sPMCA rounds, each were done in duplicate. Western blotting showed that PrP^{res} was detected in all samples at least once with the exception of the post filter sample and the filter backwash pond water.

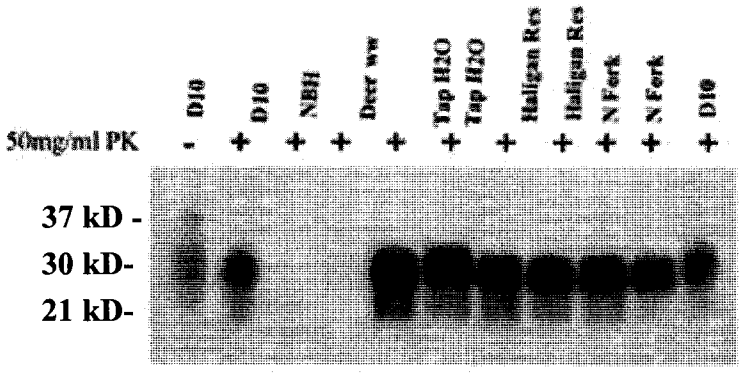


Figure 47. 6-5-07 Halligan Reservoir, north fork of the Cache la Poudre River, tap water. NBH- Normal brain homogenate negative controls. D10- Unamplified 10% infected deer brain positive controls. HT-Horsetooth reservoir water. P.River-Cache la Poudre River water. Tap- Tap water collected from Colorado State University drinking fountain. Each sample was done in duplicate (A & B). Halligan and N.fork of Cache la Poudre river samples were both positive for PrP^{res} after 5, 24hr sPMCA rounds. Tap water collected on 03-07 also showed a positive signal after 5 rounds of sPMCA.

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