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# Heat-Dependent Loss of PrP$^{res}$ Immunoreactivity Using Three Detection Systems

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**Abstract:** Prions are exceptionally resistant to physical or chemical methods of degradation. Conditions that result in prion destruction under natural conditions or that may be used for environmental decontamination or disposal of biowastes need to be elucidated. It was hypothesized that long-term heating may be effective at destroying prions. The immunodetection of protease-resistant, disease-associated Prion Protein (PrP$^{res}$) is an expedient approach to screen physiochemical conditions for potential to destroy prions. The present objective was to characterize PrP$^{res}$ immunodetectability in brain from Chronic Wasting Disease (CWD)-affected elk that was subjected to heat over time. Three commercially available diagnostic assays for CWD demonstrated progressive loss of PrP$^{res}$ immunodetectability when brain homogenates were incubated at 37, 55 and 80°C for 200 days. The rate of PrP$^{res}$ immunoreactivity reduction increased with incubation temperature and was comparatively more substantial when brain homogenates were incubated as 20% solutions in sterile water. When samples were incubated at 55 or 80°C, PrP$^{res}$ immunoreactivity eventually declined below the detection limits of all three assays. Results indicate the potential for environments and biodisposal systems that maintain high temperatures over time to naturally degrade prions. Ultimately, bioassay is necessary to determine whether infectivity is absent from treated samples with depleted, detectable PrP$^{res}$. Furthermore, findings suggest that PrP$^{res}$ immunoassays may not be accurate when applied to brain samples that were weakly positive and have had long post-mortem or post-collection intervals, or have been exposed to high temperatures.

**Key words:** Autolysis, chronic wasting disease, diagnosis, prion, transmissible spongiform encephalopathy

## INTRODUCTION

Transmissible Spongiform Encephalopathies (TSEs) are a group of fatal infectious neurodegenerative diseases that include scrapie of sheep and goats, Chronic Wasting Disease (CWD) of free-ranging and captive deer and elk, Bovine Spongiform Encephalopathy (BSE) of cattle and Creutzfeldt-Jakob Disease (CJD) of humans. The BSE agent has been implicated as the cause of variant CJD in humans (Bruce *et al*., 1997; Collinge *et al*., 1996; Will *et al*., 1996). The potential for foodborne transmission of BSE agent to humans and cases of CJD occurring in unusually young patients who had consumed venison, (CDCP, 2003; Belay *et al*., 2001; Peltier *et al*., 2002) raise concern for similar zoonosis of CWD agent (Belay *et al*., 2004). The perceived human health risk related to TSEs, especially BSE, has encouraged large economic expenditures for surveillance and containment efforts and has necessitated the establishment of enhanced food safety measures.

The causative agent of TSEs is believed to be the prion (Prusiner, 1982). Little is known about the fate of prions in the environment. Because prions are resistant to several physical and chemical decontamination methods (Taylor, 2000) and one study demonstrated the survival of scrapie agent in the environment for several years (Brown and Gajdusek, 1991), it is generally believed that these agents result in long-term ecological contamination. Prions may be introduced to an environment through shedding of the agent from live hosts, or through decomposition and scavenging of dead hosts. Environmental persistence of prions may provide an avenue for horizontal transmission to susceptible hosts (Miller *et al*., 2004). Contamination of premises with prions is a serious consequence for herds containing an animal affected with scrapie or CWD and has been suspected in some outbreaks (Miller *et al*., 1998; Sigurdarson, 1991) Factors that favor natural prion degradation within the environment need to be elucidated. Accepted methods for prion elimination, including steam-autoclaving at high

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temperatures, alkaline hydrolysis, treatment with phenolic disinfectant such as LpH and treatment with sodium hypochlorite, or sodium hydroxide, may not be accessible or practical for disposal of potentially infected animals or for decontaminating the environment (Ernst and Race, 1993; Taylor, 2000; Unal *et al*., 2006). Alternative methods for disinfecting TSE-contaminated biomaterial or surroundings are needed.

The exact nature of the prion is undetermined, however, it is at least partially comprised of a proteinase-resistant, disease-associated isoform of host-encoded Prion Protein ($PrP^{res}$) (McKinley *et al*., 1983; Prusiner, 1982). Prions can be demonstrated by immunodetection of $PrP^{res}$ or by animal bioassay. While bioassay is ultimately needed to determine if materials suspected to contain prions are infectious, laboratory animal welfare concerns and the time and expense needed to complete such studies limits its application for screening decontamination protocols.

We hypothesize that over a relatively long period of time, temperature-dependent degradation of prions is more significant than previously considered. Using a Western blot, an initial study demonstrated substantial temperature-dependent, progressive loss of detectable $PrP^{res}$ in brain samples from CWD-affected cervids that were incubated at 37, 55 and 80°C for up to 90 days (Triantis *et al*., 2007). Since $PrP^{res}$ detection has been demonstrated to correlate with infectivity (Race *et al*., 1998) and since modifications to structure or conformation of $PrP^{res}$ have been associated with reduction in infectivity (Caughey *et al*., 1997; Gasset *et al*., 1993; McKinley *et al*., 1983; Safar *et al*., 1993) evaluation of $PrP^{res}$ detectability is a convenient approach to screen for conditions that may favor prion destruction prior to conducting bioassay. To substantiate prion destruction, bioassay is needed. However, prior to undertaking the expense and the responsibilities of designing useful animal experiments, alternative explanations of the the initial findings that are unrelated to $PrP^{res}$ destruction and loss of infectivity need to be explored and ruled out. These include selective epitope loss or masking, formation of non-mobile aggregates and increased sensitivity to Proteinase K (PK) use in the test protocol.

The objective of the present study, was to demonstrate the consistency of *in vitro*, heat-dependent loss of $PrP^{res}$ immunoreactivity in brain samples from CWD-affected elk, by using 2 EIAs in addition to the Western blot. Each of these $PrP^{res}$ detection systems use different approaches for selective $PrP^{res}$ identification and employ unique antibodies. Here we confirm and extend initial findings, demonstrating consistent progressive reduction and eventual loss of detectable $PrP^{res}$ in brain samples similarly treated with heated incubation for up to 200 days and rule out several trivial explanations for the phenomenon.

## MATERIALS AND METHODS

**Animals:** Elk 1, an adult male, was found dead in a US National Park in the Rocky Mountains. Extensive accumulation of PrP was identified in the retropharyngeal lymph node, tonsil and nuclei of the obex of the medulla oblongata using immunohistochemistry. The brainstem was affected with moderate spongiform degeneration.

Elk 2, an adult female in poor body condition, died in a US National Park in the Rocky Mountains after being observed with a braced stance and lowered head. Using immunohistochemistry, extensive accumulation of PrP was detected in the retropharyngeal lymph node and nuclei of the obex of the medulla oblongata. The brainstem was affected with severe spongiform degeneration.

**Incubation:** Brain tissue from the two elk was obtained at necropsy. Caudal brainstems were homogenized separately using a homogenizer and stored at -70°C. 0.5-5 g of thawed pure brain homogenate were placed in sealed 2-5 mL microcentrifuge tubes. Homogenates from both elk were incubated in replicate trials at 55°C and homogenates from elk 1 were additionally incubated at 37 and 80°C (Table 1). At day 0 (un-incubated control) and after approximately 16, 30, 60, 90 and 200 days of incubation, aliquots were removed from homogenates to be evaluated for $PrP^{res}$ detectability using three assays. For each elk, incubation trials at 55°C were performed in duplicate; one homogenate was incubated as whole brain, whereas another was incubated as a 20% concentration of brain in sterile protease-free water. Five replicate incubation trials were completed for elk 1 and 2 were completed at 55°C for elk 2.

For each incubation trial, incubating brain homogenates were tested using 3 detection systems at 5 time-points over a period of 200 days and were compared to non-incubated control brain homogenate from the respective elk.

**$PrP^{res}$ immunodetection:** Unincubated brain homogenates from both elk contained abundant $PrP^{res}$. When evaluated with the Western blot, undiluted samples lost lane and band definition because of the abundance of $PrP^{res}$. When evaluated with the EIAs, OD values were maximized outside the linear range of enzyme detection kinetics. Therefore, control (un-incubated) and incubated samples were diluted differentially for each elk and for each $PrP^{res}$ detection system such that the effect of incubation

Table 1: Number of separate incubation trials performed at three different temperatures for each elk

| | 37°C | 55°C+ | 80°C |
|---|---|---|---|
| Elk 1 | 1 | 5 | 1 |
| Elk 2 | * | 2 | * |

+Incubation trials were performed in replicate at 55°C; 5 brain homogenates from elk 1 were incubated as whole brain and 5 were incubated as 20% concentrations of brain in sterile protease-free water. Likewise, 2 brain homogenates from elk 2 were incubated as whole brain and 2 were incubated as 20% concentrations of brain in sterile protease-free water. *No incubation trials were performed at these temperatures using tissues from elk 2

conditions on $PrP^{res}$ immunodetectibility could be accurately evaluated. Since the Western blot and EIA1 utilize PK digestion, dilutions were performed after the PK digestion in order to conform to standard assay conditions.

For the Western blot, control (un-incubated) and incubated brain homogenates were diluted to 10% solutions with 1X kit homogenization buffer (final concentration being equivalent for homogenates incubated as whole brain versus those incubated as 20% aqueous solutions) and then digested with PK per manufacturer's protocol (10 uL 100 $uL^{-1}$ digestion buffer and 1.8 Units $mL^{-1}$ PK at 48°C for 40 min). Control (un-incubated) and incubated brain homogenates were further diluted at 1/6 (elk 1) and 1/2 (elk 2) with sterile protease-free water following PK digestion. These dilution levels are those which produced the strongest signal without impeding protein bands within adjacent lanes and were below the maximum detection limit of the densitometer. Homogenates were mixed 1:1 with SDS sample buffer prior to electrophoresis. To complete the Western blot, a commercially available kit was used per manufacturer's protocol. $PrP^{res}$ signal intensity was estimated using densitometric analysis of all three CWD-specific $PrP^{res}$ bands. Percent $PrP^{res}$ signal reduction was estimated by comparing intensity of $PrP^{res}$ immunoreactivity between control (un-incubated) homogenates and incubated homogenates at each time-point. For incubation trials performed at 55°C, the mean percent $PrP^{res}$ signal reduction was calculated for each elk.

EIA 1 is a sandwich ELISA that uses two monoclonal antibodies and, similar to the Western blot, requires a PK digestion step. For EIA 1, PK digestion was performed on incubated samples according to manufacturer's protocol. Following PK digestion, control (un-incubated) and incubated brain homogenates were diluted at 1/20 (elk 1) and 1/137 (elk 2) with bovine brain homogenate. These dilution levels were those that produced the most repeatable OD values within the linear range of enzyme detection substrates. The final concentrations were equivalent for homogenates incubated as whole brain versus those incubated as 20% aqueous solutions. Brain homogenate was used for dilution in order to maintain a consistent test matrix. Bovine brain tissue was negative for BSE by all tests used by the present study. Prior to testing, each sample was prepared as an 11% brain homogenate solution in 5% glucose (kit homogenization buffer). The remaining sample preparation was performed according to manufacturer's protocol.

EIA 2 is an antigen capture ELISA that uses a single monoclonal antibody. This second generation, conformation-dependent assay utilizes the differential binding of aggregated $PrP^{res}$ to Seprion® affinity ligands and thus is not dependent on PK digestion (Triantis *et al*., 2007) EIA 2 was completed per manufacturer's protocol. Prior to testing, each sample was prepared as an 11% brain homogenate solution in sterile protease free water. Control (un-incubated) and incubated brain homogenates were diluted at 1/261 (elk 1), or 1/137 (elk 2) with 11% negative bovine brain homogenate. As for EIA 1, these dilution levels were those that produced the most repeatable OD values within the linear range of enzyme detection substrates and the final concentrations were equivalent for homogenates incubated as whole brain versus those incubated as 20% aqueous solutions.

For each EIA, OD values were used to represent $PrP^{res}$ signal intensity. Percent $PrP^{res}$ signal reduction was estimated by comparing $PrP^{res}$ signal intensity between control (un-incubated) homogenate and incubated homogenate at each time-point. In addition, for incubation trials performed at 55°C, the mean percent $PrP^{res}$ signal reduction was calculated for each elk. Positive or negative test results were determined by comparing a sample's OD value to OD cut-off values that are established for each test run using control materials provided by the test kits.

## RESULTS

Prior to incubation (day 0), homogenized brain tissue from both elk was positive for CWD by Western blot, as indicated by the presence of three distinct protein bands which represent glycosylated forms of truncated $PrP^{res}$. Likewise, when using either EIA, unincubated, homogenized brain tissue from both elk was consistently positive for CWD as indicated by an OD value for $PrP^{res}$ immunoreactivity that exceeded cut-off OD values.

$PrP^{res}$ immunoreactivity as indicated by each detection system was considerably reduced over time in samples from Elk 1 that were incubated at 37, 55 and 80°C (Fig. 1 and 2).

Numbers at the top of lanes indicate days of incubation. M denotes lane containing molecular

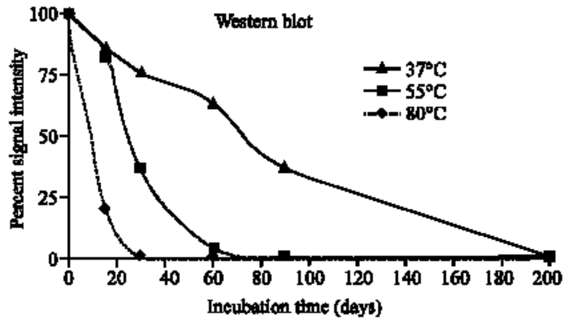


Fig. 1: Western blots of homogenized brain tissue from elk 1 incubated for 0 to 200 days at 37, 55 and 80°C and diluted 1/6 in sterile protease free water prior to testing. A = sample incubated at 37°C. B = sample incubated at 55°C. C = sample incubated at 80°C

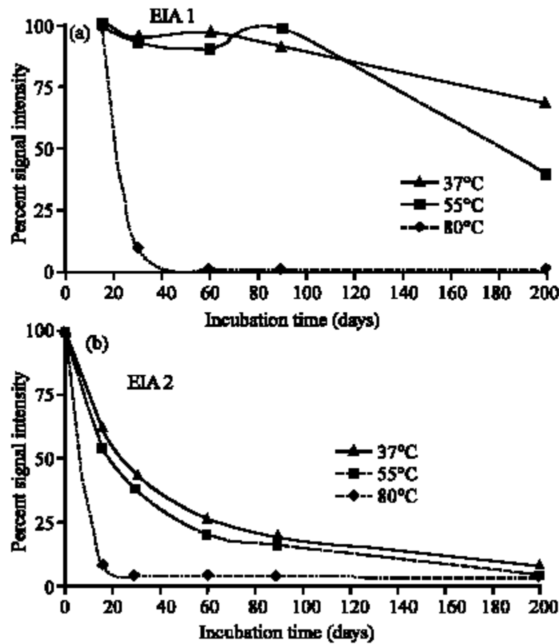


Fig. 2: Percent PrP$^{res}$ signal reduction as detected by 3 assays when applied to homogenized brain tissue from elk 1 incubated for 0 to 200 days at 37, 55 and 80°C

weight markers (kDa) and non-protease-truncated PrP (homogenous band spread between 25-35 kDa labeled with asterisk). Samples positive for CWD contain 3 distinct bands (white arrows) starting at 30 kDa that represent glycosylated forms of PrP$^{res}$. The bands at 31 kDa (black arrow) result from nonspecific binding of the secondary antibody to proteinase K. Note progressive loss of intensity for CWD-specific immunoreactivity in all samples over time.

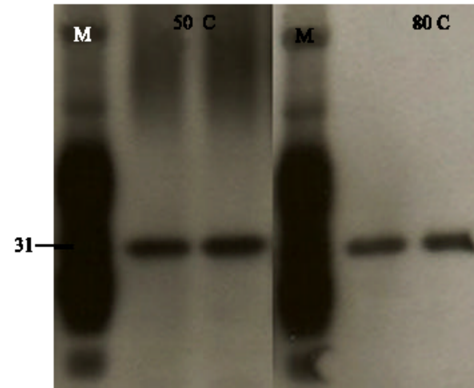


Fig. 3: Western blots of homogenized brain tissue from elk 1 incubated for 0 to 200 days at 55 and 80°C

Signal loss reflected by panel A corresponds to reduction in band density as determined with densitometry applied to the Western blot portrayed in Fig. 1. Signal loss displayed in panels B and C represents reduction in optical density values. Incubated brain homogenates were differentially diluted for each assay prior to testing: WB-1/6 in sterile protease-free water; EIA 1-1/20 in BSE-negative bovine brain; EIA 2-1/261 in BSE-negative bovine brain.

At all temperatures, Western blots failed to produce PrP$^{res}$ bands when applied to tissues incubated for 200 days. Furthermore, PrP$^{res}$ bands were not evident in Western blots of samples that were incubated at 55 and 80°C for 200 days and were not diluted following incubation and PK digestion (Fig. 3). When samples were tested with EIA 1, OD values for PrP$^{res}$ immunoreactivity were reduced below the cut-off for a positive result after incubation at 80°C for 30 days. Although OD values of samples incubated at 37 and 55°C were substantially reduced over time, the OD values remained above the cut-off value after incubation for 200 days. When samples were tested with EIA 2, OD values for PrP$^{res}$ immunoreactivity were reduced below the cut-off for a positive result after incubation at 80°C for 16 days, or 37 or 55°C for 200 days. The rate of PrP$^{res}$ immunoreactivity loss as demonstrated by each detection system increased with incubation temperature. PrP$^{res}$ immunoreactivity exhibited by all detection systems for the sample incubated at 80°C was greater than 80% reduced by day 30 and greater than 95% reduced by day 60. PrP$^{res}$ immunoreactivity as evidenced by all detection systems was substantially reduced over time as a result of incubation at 37 and 55°C, however, the rate of immunoreactivity reduction was less notable when samples were analyzed with EIA 1 versus the other 2 detection systems.

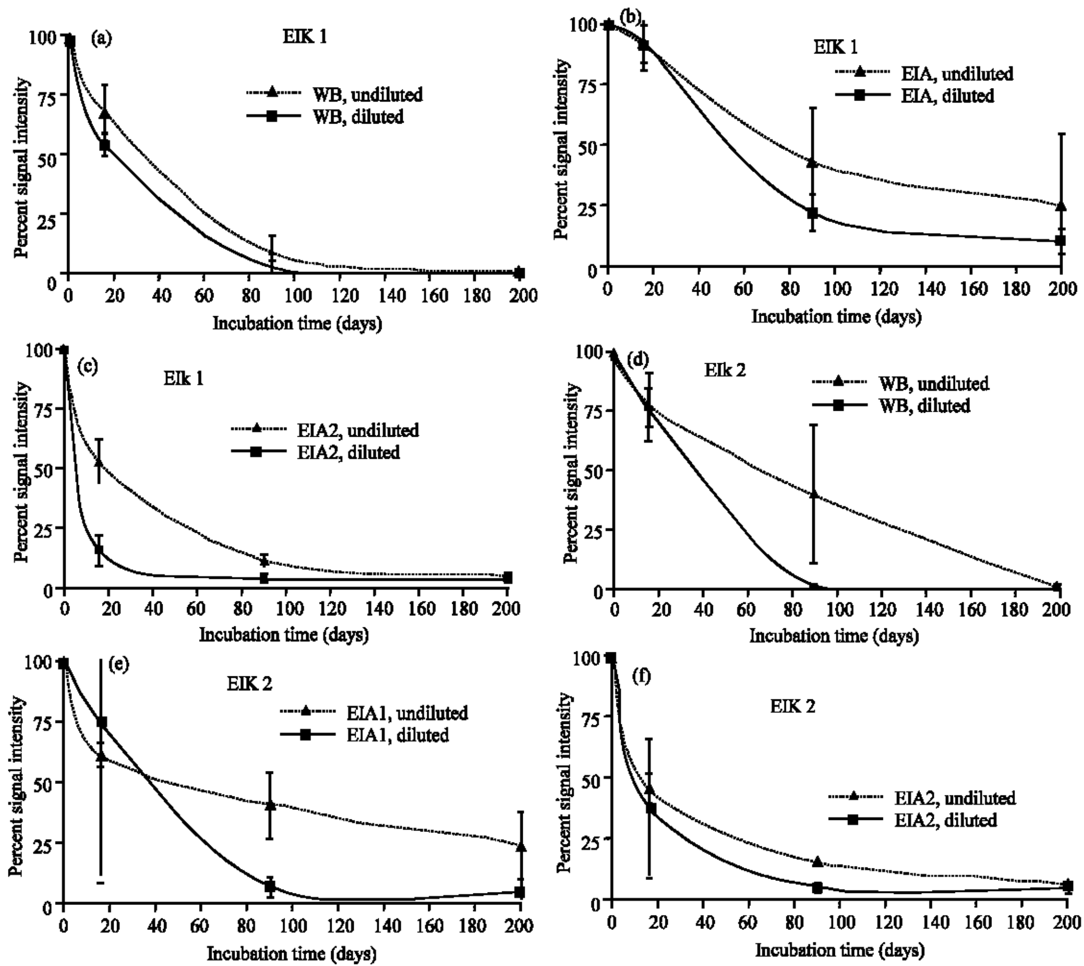


Fig. 4: Mean percent $PrP^{res}$ signal reduction as detected by Western blot and immunosorbent assays when applied to homogenized brain tissue from elk 1 and elk 2 incubated for 0 to 200 days at 55°C

Homogenates were not further diluted prior to testing. M denotes lane containing molecular weight markers (kDa) and non-protease-truncated PrP. Note that both samples lack CWD-specific immunoreactivity.

To more precisely characterize the effect of heated incubation on $PrP^{res}$ detectability, multiple incubation trials were completed at 55°C (a temperature relevant to mortality composting) using brain tissue from both elk. A similar loss in $PrP^{res}$ immunoreactivity was demonstrated over time when evaluating tissue from both elk with each detection system (Fig. 4). Further, the rate of $PrP^{res}$ immunoreactivity loss was greater for samples that incubated as 20% aqueous solutions. Homogenates incubated as whole brain from both elk evaluated with the Western blot produced $PrP^{res}$ bands that were variably noticeable after 90 days of incubation and were always diminished after 200 days of incubation. Whereas most homogenates incubated as 20% aqueous solutions from both elk evaluated with the Western blot were devoid of $PrP^{res}$ bands following 90 days of incubation. When homogenates incubated as whole brain were evaluated with EIA 1, OD values of samples from elk 1 were inconsistently reduced below the cut-off for a positive result following 90-200 days of incubation and were reduced below the cut-off in all samples from elk 2 after 16 days of incubation. OD values of homogenates incubated as 20% aqueous solutions from elk 1 were inconsistently below the cut-off after 200 days of incubation. OD values of homogenates incubated as 20% aqueous solutions from elk 2 were inconsistently below the cut-off after 16 days of incubation, but were always below the cut-off after 60 days. When homogenates incubated as whole brain of both elk were evaluated with EIA 2, OD values were inconsistently reduced below the cut-off for a positive result on the $90^{th}$ day of incubation and were always below the cut-off after 200 days of

incubation. OD values were below the cut-off in all homogenates incubated as 20% aqeous solutions from both elk that were incubated for 90 days and evaluated with EIA 2.

Signal intensity loss represents reduction in band density as determined with densitometry (immunoblots) or reduction in optical density values (immunosorbent assays). Brain homogenates were incubated as whole brain, or as 20% aqueous solutions. After incubation, homogenates were differentially diluted for each assay prior to testing: WB-1/6 (elk 1), 1/2 (elk 2) in sterile protease-free water; EIA 1-1/20 (elk 1), 1/137 (elk 2) in BSE-negative bovine brain; EIA 2-1/261 (elk 1), 1/137 (elk 2) in BSE-negative bovine brain. Data portrayed for elk 1 represent a mean of 5 trials. Data presented for elk 2 represent a mean of 2 trials.

## DISCUSSION

Prions are resistant to most conventional decontamination procedures. Methods that have been demonstrated to inactivate prions are often times not practical, available, or cost-effective for disposal of biowastes from TSE-infected animals. Novel disposal and decontamination techniques that prevent or eliminate environmental prion contamination are needed. High-temperature autoclaving has demonstrated inactivation of prions (Taylor, 2000). Should prion decomposition also occur at a slower rate under more moderate heat conditions, there is potential for prion degradation by biowaste disposal systems that maintain temperature for sustained time periods, such as livestock mortality composting,. Using Western blot, a recent study demonstrated loss or reduction of immunodetectable $PrP^{res}$ in tissues from scrapie-affected sheep that were composted for up to 148 days at temperatures periodically above 60°C (Huang *et al*., 2007). The present study reveals a similar reduction in detectable $PrP^{res}$ that was a result of exposure to heat alone, without the presence of complex proteolytic systems maintained by microbial consortia.

Using a Western blot test system, we previously demonstrated progressive loss of detectable $PrP^{res}$ from homogenized brain samples of CWD-affected cervids that were incubated at 37, 55 and 80°C for up to 90 days (Triantis *et al*., 2007). The present study substantiates these findings using two additional, dissimilar $PrP^{res}$ detection systems and rules out several trivial explanations for the $PrP^{res}$ disappearance Each detection system displayed progressive loss of $PrP^{res}$ immunoreactivity over time, notable even on the shortest incubation increment (16 days), regardless of incubation temperature. Furthermore, the rate of $PrP^{res}$ immunoreactivity loss increased with incubation temperature. Over time, immunoreactivity was observed to decline below the detection limits of all three detection systems when samples were incubated at 55 or 80°C and declined below the detection limits of the Western blot and EIA 2 when incubated at 37°C. At 55°C, all $PrP^{res}$ detection systems demonstrated that samples incubated at 55°C as 20% aqueous solutions had accelerated loss of detectable $PrP^{res}$ relative to samples that were incubated without dilution.

The use of $PrP^{res}$ detection systems with differential methods for selective $PrP^{res}$ identification was needed to clarify initial findings, as diminished $PrP^{res}$ immunodetection can occur without $PrP^{res}$ degradation. For example, selective epitope loss or masking may preclude immunodetection of $PrP^{res}$. This seems an unlikely explanation for the present results given that immunodetection was similarly influenced by experimental conditions when using detection systems employing different antibodies. Second, the formation of oligomeric $PrP^{res}$ aggregates may reduce the amount of antigen that is available to immunodetection systems. While this occurrence could provide an explanation for reduction in $PrP^{res}$ immunoreactivity evidenced by Western blot, particularly for samples that have high molecular weight smears of immunoreactive material, aggregate formation should not affect the immunodetection of either EIA used by the present study. Finally, experimental conditions may have increased susceptibility of $PrP^{res}$ to PK. However, this is unlikely as EIA 2 does not use a PK digestion step and as $PrP^{res}$ immunoreactivity was similarly reduced in non-digested samples evaluated with the Western blot. For these reasons, the authors assume that loss of $PrP^{res}$ immunoreactivity represents $PrP^{res}$ degradation. Further study is needed to elucidate the mechanisms potentially accountable for $PrP^{res}$ degradation.

Prior to testing, it was necessary to differentially dilute brain for each elk and $PrP^{res}$ detection system; therefore, direct comparisons regarding the performance of each assay under the experimental conditions could not be made. However, diagnosticians using $PrP^{res}$ detection systems for CWD diagnosis should be aware that negative test results may have questionable accuracy when derived from brain samples which were subjected to heat over time. This is especially a concern for animals that are in a stage of disease where $PrP^{res}$ accumulation is marginally above a test's detection limits. If exposure conditions are

associated with severe brain tissue decomposition, the validity of a negative test result is further jeopardized by the inability to properly identify the appropriate sub-gross anatomic site for testing.

## CONCLUSION

The present study used $PrP^{res}$ immunodetection as a practical approach for assessing a potential prion-degradation effect and ruling out various confounding phenomena that could result in loss of $PrP^{res}$ signal. Such an approach is a necessary precursor to meaningful animal bioassays. We speculate that the time and temperature-dependent reduction in $PrP^{res}$ immunodetection suggests that such experimental conditions could reduce infectivity. Given the present findings, bioassay is warranted to confirm loss of Infectivity from samples that are depleted of detectable $PrP^{res}$. If reduction of infectivity can be demonstrated, it is possible that natural, heat-producing microbial systems can be developed and exploited to decontaminate prion-containing materials.

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