ABSTRACT

The transmissible spongiform encephalopathies (TSE) are a group of neurological prion diseases of mammals caused by infectious prion proteins. Prions may be present in raw wastewater through the discharge, animal rendering and meat processing operations that may process BSE-contaminated cattle or scrapie infected sheep. Recently, a cell culture method was developed to detect the infectivity of prions in animal tissues.

This study developed a method for extracting prion proteins from Class B biosolids and evaluated the survival of mouse scrapie prion proteins in Class B biosolids at 37°C and 60°C using an enzyme linked immunospot assay. A method was developed using 4 M urea (held at 80 °C for 10 minutes) to extract the prions from the biosolids followed by reduction of the urea concentration using membrane centrifugation. The efficiency of recovery of infectious prions from the biosolids was 17.2%.

A 2.43-log_{10} reduction in prion infectivity was observed under mesophilic (37°C) temperatures after 15 days and a 3.41-log_{10} reduction after 10 days under thermophilic conditions (60°C). Reduction was greater in the biosolids than controls in phosphate buffered saline, suggesting factors other than temperature were also playing a role in the loss of infectivity of the prions in the biosolids.
INTRODUCTION

Literature Review

Incidence of Prion Related Disease:

In 1986, the first case of bovine spongiform encephalopathy (BSE), also known as mad cow disease appeared in Britain. BSE may have arisen from an infection with scrapie obtained from infected sheep. The numbers of cases have dramatically increased since 1986 due to the feeding of processed offal from cattle and other animals including sheep to cattle. There was a peak in the number of cases in 1992. In 1988, legislation was introduced that banned the feeding of any ruminant-derived protein to other ruminants. The initial ban was enlarged and extended in various ways such as a specified bovine offal ban and a meat and bone meal ban. Because of these regulations, the occurrence of cases of BSE has decreased since 1992.

Prions (short for proteinaceous infectious particle) can cause scrapie, Creutzfeldt-Jakob disease, and other diseases. The transmissible spongiform encephalopathies (TSE) are a group of neurological prion diseases of mammals, including Kuru in humans, scrapie in sheep and goats, Creutzfeldt-Jakob disease (CJD), sporadic Creutzfeldt-Jakob disease (spCJD), and new variant Creutzfeldt-Jakob disease (nvCJD) in humans and bovine spongiform encephalopathy (BSE) in cattle (Gale 2006). Also, TSE affect deer, elk, and moose (chronic wasting disease [CWD]) and ranch raised mink (transmissible mink encephalophathy [TME]). TSE cause dementia (loss of intellectual abilities or ataxia) and loss of muscle control during voluntary movement. These symptoms result from the progressive loss of brain function. TSE do not stimulate an immune response so antibodies are not produced. As a result, TSE are progressive diseases and always lead to death (Strauss and Strauss, 2008).

Kuru is a human disease causing progressive ataxia. In 1957, there was a Kuru epidemic in New Guinea because of the practice of ritualized cannibalism and 1% of the population was diagnosed. Persons who ate brains of relatives in a ritualistic feast were more likely to be infected with Kuru (Strauss and Strauss, 2008).

Historically, scrapie has been the most common TSE in animals (Saa et. al., 2006). Sheep scrapie is unique because epizootics can be sustained by horizontal animal-to-animal transmission (Johnson et. al., 2006). Sheep and goats are susceptible to natural scrapie, which has been around for centuries. So far, there is no evidence that scrapie has spread to humans, although humans may have consumed scrapie-infected sheep during the past two centuries (Strauss and Strauss, 2008).

The apparent differences in the transmissibility of bovine and sheep prions to humans can be better understood when considering the disease governing interspecies transmission of prions and the merging of certain prion strains with new prion disease phenotypes (Peretz et. al 2002). While the ability of interspecies transmissions of infective prions have been researched, the appropriate treatment methods should be standardized for infectious prions considering the possibility of transmission between animals and humans.
BSE is of considerable public health concern because it has been associated with nvCJD; both of these diseases have no known cure and symptoms can be subclinical for many years. The classic form of CJD was first reported in the 1920s. About 10%–15% of CJD cases are associated with abnormal mutations of the prion protein. In addition, 85% of classic CJD cases occur as a sporadic disease with no recognizable pattern of transmission (Belay 2005). Occupational infections have not been recorded from working with prions. There has been no increase in incidence of CJD among pathologists who encounter cases of the disease. Additionally, prions are not airborne, so ambient air transports are not an issue in disease transmission.

Sporadic creutzfeldt-Jakob Disease (spCJD) appears to arise spontaneously due to the instantaneous mutation of the normal prion protein (PrP\(^C\)). SpCJD can be transmitted through zoonotic exposure both directly and through diet. It appears to occur worldwide, with an incidence of one case per million people. Most cases involve older adults in the 50 to 70 year age group (Prusiner 2004).

New variant Creutzfeldt-Jakob Disease (nvCJD) is a new type of CJD that has been reported in the United Kingdom (U.K.) (Belay 2005). Usually, TSE are characterized by an extremely long incubation period. For example, Kuru may take 40 years to develop symptoms after infection (Saa et al., 2006). However, research demonstrated that the incubation time of diagnosed BSE and nvCJD was the same in two different experimental mice strains (Belay 2005). The first cases of nvCJD occurred in young adults, with 12 cases reported in 1995 and 1996. These cases were unusual because they occurred in the teens. The diagnosis of this disease was quite unique because a halo of intense spongiform degeneration appeared in the brain. This is not a typical symptom of CJD in the United States. Animal experiments were performed using macaque monkeys and marmosets with bovine prions. Only the macaques exhibited similar symptoms to those found in nvCJD (Prusiner 2004). The specific geographic occurrence and chronology of nvCJD showed that BSE prions can be transmitted to humans. The total estimated number of cattle potentially infected with BSE was over 2 million. Approximately 750,000 BSE infected cattle were slaughtered and consumed by millions of U.K. residents between 1980 and 1996 (Belay 2005). The number of cattle confirmed with BSE in the U.K. was more than 180,000 in 2003. TSE in humans have been the subject of increased attention and concern. The known consumption of contaminated meat and concerns about cross contamination of cattle feed with prohibited material (meat and bone meal [MBM]) intended for other species prompted the introduction of a specified bovine offal ban by the Food Standard Agency in the U.K. in 1990. After the prohibition of the use of any animal protein in feed for any farmed animal species in Britain in 2001, the reported cases per year dropped dramatically (Strauss and Strauss, 2008).

In the U.S., no cases of nvCJD have been linked to the consumption of American beef. The U.S. Department of Agriculture (USDA) continuously monitors cattle to identify disease and to prevent infectious animals from entering the human food chain. In the U.S., there was less than 1 case of BSE infection per one million adult cattle based on adult cattle population of 42 million animals between 2004 and 2006 (USDA 2006). To control the spread of scrapie in the U.S. as well, the Animal and Plant Health Inspection Service (APHIS) and the USDA set administrative regulations in the Code of Federal Regulations (9 CFR, Part 79) which restricts the interstate movement of diseased sheep and goats to prevent the spread of scrapies.
Characteristics of Prions:

Cellular prion is a normal protein found on cell membranes. It has about 250 amino acids (M.W. ~36kDa), and is located on the short arm of chromosome 20 in humans. It has a mainly alpha-helical structure and is also largely hydrophobic. Cellular prion protein has a role in the renewal of hematopoietic stem cells and the development of neurons. It also works as a copper-dependent antioxidant (Brown et. al., 1999). However, the role of prion protein is not fully understood.

A prion is a type of infectious agent composed only of protein. Prion disease is transmissible by inoculation or ingestion of infected tissues or homogenates. The particles are present at high levels in the brain or other central nervous system tissues, and at slightly lower levels in lymphoid tissues including the spleen, lymph nodes, gut, bone marrow and blood. Although the biochemical nature of infectious prions is not yet understood, their infectivity is strongly associated with the presence of abnormal prion protein structure.

Complete denaturing of infectious prion has not yet been achieved (Weissmann et. al., 2002). There are technical difficulties when experimenting with prion agents due to their long incubation period (more than 10 years in some cases), and because they are expensive to maintain in animals (Strauss and Strauss, 2008). The infectious agent that transmits prion disease exhibits extraordinary resistance to inactivation in comparison to conventional pathogens including dry heat at temperatures under 600°C, exposure to ionizing, ultraviolet, and microwave radiation, protease treatment, contact with most chemical disinfectants, boiling, and autoclaving under standard conditions (Xin et. al., 2007).

Prions were initially thought to be viruses. However, prions are distinct from viruses. Prions are composed of an alternative isoform of a cellular protein while viruses are composed of viral proteins that are encoded by a viral genome (Prusiner 2004). In contrast to viruses, prions do not have a genetic component such as DNA or RNA.

The cellular isoform of prion protein (PrP\textsuperscript{C}) is found on cell membranes of healthy people and animals, although its function has not been fully resolved. The infectious isoform of PrP\textsuperscript{C} is known as the scrapie prion protein (PrP\textsuperscript{Sc}). Also, the misfolded form of the prion protein is known as PrP\textsuperscript{TSE}. PrP\textsuperscript{TSE} is a broader term of infectious prion protein causing TSE. It is widely accepted as a biomarker for prions and generally considered infectious. Prion infectivity changes with PrP\textsuperscript{TSE} aggregate size and PrP\textsuperscript{TSE} are more likely to stay aggregated.

The PrP\textsuperscript{Sc} isoform of the protein has a greater β-sheet content, rather than the α-helical form of PrP\textsuperscript{C}. PrP\textsuperscript{C} has approximately 42% α-helix and 3% β-sheet character, while PrP\textsuperscript{Sc} is rich in β-sheets (43%) with a diminished α-helix content (30%)(Xin et. al., 2007). PrP\textsuperscript{Sc} is used to designate all abnormal, pathogenic PrP, and is the only identified component of the infectious prion modified form of PrP\textsuperscript{C} (Glatzel 2004). Infectious prions can disrupt the normal tissue structure. When endogenous PrP\textsuperscript{C} has contact with PrP\textsuperscript{Sc}, conversion from PrP\textsuperscript{C} to PrP\textsuperscript{Sc} occurs, and PrP\textsuperscript{Sc} accumulates in the body. The conformational change from PrP\textsuperscript{C} to PrP\textsuperscript{Sc} is believed to be rare without interaction with PrP\textsuperscript{Sc}. However, once PrP\textsuperscript{Sc} is formed, it induces the formation...
of more PrP<sup>Sc</sup>. After an unknown incubation period, holes in the brain tissue will sometimes be formed with a resultant spongy architecture due to the loss of neurons.

PrP<sup>Sc</sup> is resistant to digestive inactivation with proteases such as Proteinase K at 37°C for 1 hour, while PrP<sup>C</sup> is completely destroyed by the treatment. Proteinase K truncates PrP<sup>Sc</sup> and the longest remaining sequence is about 142 amino acids long. As a result, the smaller protease-resistant prion protein (PrP<sup>res</sup>) remains after the treatment (Strauss and Strauss, 2008).

**Proteinase K (PK) Digestion:**

Proteinase K (PK), a serine protease, can be used to purify PrP<sup>Sc</sup> because abnormal prion protein is not totally degraded by the enzyme. Western blot analysis showed bands of both healthy brain homogenate (hBH) and infected brain homogenate (iBH) by hamster-adapted scrapie between 27-35 kDa without PK treatment. A shorter band, 27-30 kDa, indicated PrP<sup>Sc</sup> core size that is only found in an infected brain homogenate sample after PK treatment (Leita et al., 2006).

**Environmental Soil Contamination of PrP<sup>Sc</sup> by Sewage Sludge:**

Biosolid application to land is a method to recycle natural resources and beneficial for the environment. Sewage sludge is the solid, semi-solid, or liquid generated during the treatment of domestic sewage (Pepper et al., 2007). There are several ways prions can enter into biosolids, including animal rendering and meat processing operations that may process BSE-contaminated cattle or scrapie sheep. Also, there is the possibility of discharged contaminated urine, feces and blood from CJD or nvCJD patients. Most conventional wastewater treatment (WWT) facilities rely on anaerobic digestion to reduce the mass and volume of activated sludge biomass production. The conventional treatment of sewage sludge, mesophilic anaerobic digestion, was reported to not reduce prions numbers due to the extremely resistant properties of prion proteins (Glen et al., 2008). Studies indicate that some bacteria such as ruminal microflora of cattle have the potential to degrade PrP<sup>Sc</sup>; however, prion infectivity tested by in vivo bioassay of Strain hamsters still remain even in the absence of a Western blot signal (Scherbel et al., 2007).

Gale et al. (2001) conducted quantitative risk assessment for BSE in sewage sludge. The exposure of biosolids to grazing cattle was calculated as 49.52 BSE cases/year out of 700,000 cows by application of treated sewage sludge to land when 1% of brain/spinal cord of over thirty month scheme (OTMS) carcasses at the abattoir entered the sewer system. OTMS was introduced in the U.K. in 1996 as a management control for the slaughter and disposal of cattle over the age of 30 months. Realistically, only 0.01% could enter the wastewater stream according to their assumption, which would reduce the number of BSE cases to 1.00 BSE per 1,400,000 cows. The exposure to humans through vegetable crops grown by treated sewage sludge is much lower. The risk is 1.32x10<sup>-9</sup> person infected/year in the 0.01% sewage entry category (Gale et al. 2001). This research indicated the possibility of manure causing contamination of cows by the use of land applied treated sewage sludge.

The study by Gale et al. (2001) was focused on practices in England and Wales which may not apply to practices in the United States. There are spatial and temporal variations in the
amount of sludge applied onto land. The model used by Gale et al. (2001) has several assumptions including that there is no destruction of the BSE agent during sewage sludge treatment (anaerobic digestion), no decay in the soil, and no threshold dose for the consumption of soils. The number of 700,000 cattle/year was calculated based on the 4.5 year average number of cows that are slaughtered at abattoirs in England and Wales. The numbers of slaughtered cattle are different in the United States. While the current research did not directly quantity the actual amount of prion found in sewage sludge, it emphasized the importance of research assessing prion contamination of biosolids in the United States.

Infectivity of Prion Protein following contact with soil or activated sludge:

PrP<sup>Sc</sup> adsorbs to soil components such as clays and organic colloids. In one study, prions were found to persist in soil for more than three years. Thus, it has been proposed that soils may be a prion reservoir (Xin et al., 2007). The N-terminal region of prions associates with negatively charged soil particles by electrostatic interaction. Protein adsorption depends on the pH with differing degrees of interaction and conformational change occurring in relation to its isoelectric point (pl). The pl defines the point that the virion exists in a state of net zero charge (Gerba 1984). When the pH increases from 3.0 to 4.0, the aggregation of PrP<sup>Sc</sup> increases dramatically. The aggregation size of PrP<sup>Sc</sup> between pH 4 and 6 is relatively constant and results in the largest aggregates. When the pH increases to 8, the size of aggregation decreases (Xin et al., 2007). PrP<sup>Sc</sup> has been shown to absorb to quartz sand most tightly at pH 4, which is near the average pl of 4.6, due to the hydrophobic attraction between PrP<sup>Sc</sup> and quartz (Xin et al., 2007). The hamster adapted scrapie strain showed peaks of pl between 3.8 and 6.5, and a major peak at 4.07 (Schmerr et al., 1998). Other physiochemical properties of prions and the characteristics of the soil particle surface affect electrostatic and hydrophobic interactions (Cooke et al., 2006).

The clay mineral montmorillonite (Mte) is a common soil mineral that binds to the TSE agent (Johnson et al., 2007). TSE agents are likely to be retained in soil for long periods and soil types may affect their persistence. Johnson et al. (2007) observed that Mte binds to infected brain homogenate (BH) and that Mte-bound prions retain higher infectivity by observation of clinical symptom in Syrian hamsters. They examined the effect of Mte on the oral transmissibility in each hamster with infected BH (30µL, 3µL and 0.3µL) for a two-hour incubation time that allowed sorption of the agent with Mte. After 300 days, the numbers of animals surviving treated with Mte+ BH were less than the numbers of hamsters treated with only BH. Interestingly, sorption of the 0.3µL BH to Mte increased the transmission and disease development and shortened the incubation period. Sorption of prion proteins to Mte dramatically enhanced the rate of transmission compared to BH alone. The lowest prion protein concentration showed a larger difference in the transmission of TSE than the higher concentrations. For the 0.3µL BH, 15 out of 16 hamsters were alive after 350 days for; however, for Mte with 0.3µL BH, 10 out of 16 hamsters, including one non-TSE intercurrent death, survived after 350 days (Johnson et al., 2007).

Another oral infectivity assay was conducted using Syrian hamsters with activated sludge by Glen et al. (2008) Infected brain homogenate (0.1% w/v) was spiked into 1mL of activated sludge and fed to hamsters with activated sludge solids to determine if the brain homogenate adsorbed onto the solids was infectious. For the positive control, 0.1% of infected
brain homogenate was also fed to the hamsters. They found that the disease incubation period of prion infectivity in the bioassay (the onset of clinical symptoms) was approximately the same (between 150 days to 353 days) for the sludge solids and the positive controls. This research showed that prion protein infectivity is still retained even following contact with activated sludge solids (Glen et. al. 2008).

These observations indicate that there is a potential risk of prion ingestion by animals or even humans through land applied biosolids or soil posed by grazing or eating crops and home garden vegetables. The risk to humans through the consumption of vegetables crops is acceptably low. On the other hand, the risks to cattle are greater because they are exposed to the soil over a longer time period during grazing (Gale et. al., 2001). Thus, environmental transmission of prions could potentially result in the horizontal transmission of TSE (Leita et. al., 2006).

**Prion Protein stability in Wastewater Treatment Processes:**

A study was conducted on prion stability in wastewater treatment (WWT) processes by Kirchmayr et. al. (2006). Anaerobic digested sludges were spiked with PrPSc and incubated under mesophilic (35°C) and thermophilic (55°C) conditions. Samples were analyzed by the intensity of the luminescence on by Western blot assay. No PrPSc reduction was observed under mesophilic conditions. On the other hand, samples under thermophilic conditions declined by 20-40% of the initial values of luminescence after 302 hours. Under mesophilic conditions, PrPSc still remained detectable in the anaerobic sludge, suggesting that prion protein remained stable. (Kirchmayr et. al., 2006). However, this method did not assess infectivity.

**Prion Extraction from Soil:**

Glen et. al. (2008) assessed various detergents to optimize PrP\textsubscript{TSE} recovery from activated and anaerobic digester sludge solids. They used TSE-infected hamster brain homogenate (infected by hamster-adapted transmissible mink encephalopathy) for their study. Two concentrations (1% and 10% w/v) of each of the following four detergents were used: Sodium dodecyl sulfate (SDS), Sodium undecyl sulfate (SUS), Trion X-100, and Sarkosyl. These are widely used for protein denaturation and solubilization. PrP\textsubscript{TSE} was efficiently recovered using 1% SDS and 10% SUS from both activated and anaerobic digester sludge. The PrP\textsubscript{TSE} recovery was about 71% from the anaerobic sludge using 1% SDS.

**Scrapie Cell (SC) Assay for PrPSc:**

Western blot and immunoblot assays are useful in assessing the presence of PrPSc qualitatively while animal bioassays are a common method to study the transmission of PrPSc (Mahal et. al., 2008). However, a cell culture method was recently developed to detect the infectivity of prions in animal tissues and potentially environmental samples (Klöhn et. al., 2003). The cell culture method is easier, faster and less costly than the use of laboratory animals. The assay can be completed in two weeks compared to the most rapid mouse bioassay that requires 20 weeks (Klöhn et. al., 2003). The scrapie cell (SC) cell culture assay was introduced by Klöhn et al. (2003). Defined numbers of cells are filtered onto membranes of enzyme linked
immunospot (ELISPOT) plates and attach onto each well. ELISPOT is an immunological assay based on the enzyme linked immunosorbent assay (ELISA). ELISA technology can detect the presence of an antibody or antigen in a sample, so it can be used to determine the serum antibody level quantitatively.

Responsiveness of cells to various prion strains is different for each type of cell (Mahal et al., 2007). The mouse neuroblastoma cell line (N2a) is susceptible to certain strains of mouse prions such as the mouse-adapted Rocky Mountain Laboratory (RML) scrapie strain and commonly used for a prion research by a cell culture assay. N2a cells are infected with brain homogenate of RML strain, and change to scrapie-infected neuroblastoma cell line (ScN2a) cells as they are infected. After 15-20 days of incubation, the amounts of PrPSc increases in ScN2a cell lines. Propagation of prion proteins has been successfully performed by the SC assay method by the N2a cell line. (Klöhn et al., 2003). There are two subgroups of N2a cells: N2a-PK1 and N2a-R33. N2a-PK1 is more susceptible to the RML scrapie strain than is R33. N2a CAD-2A2D5 (CAD5) cells were derived from Cath.a-differentiated (CAD) neuronal cells. They are also highly susceptible to prion strains RML, Me7, 301C and 22L.

The RML scrapie used in this research is a Biosafety Level 2 agent, which can be a potential hazard to personnel and the environment. All work involving PrPSc should be performed in a biological safety cabinet. Laboratory personnel are required to receive appropriate training on the potential hazards associated with this work including laboratory safety training and bloodborne pathogens training. Prion research has established that prions can be denatured at 134°C for 18 minutes in a pressurized steam autoclave (Weissmann et al., 2002). Any equipment, reagents or material that cannot be autoclaved is disinfected in a 40% bleach solution for a minimum contact time of 60 minutes, and discarded in a biohazard bag.

Problem Definition:

The purpose of this research was to determine the survival of mouse prion proteins in Class B biosolids, using a quantitative analysis of PrPSc and to develop prion extraction methods from biosolids. This assay combines a cell culture technique with an immunospot assay.

MATERIALS AND METHODS

Biosolids

Class B biosolids were collected from the Ina Road Wastewater Treatment Plant located in Tucson, Arizona. The biosolids were produced from a mesophilic anaerobic digester with a retention time between 25 to 30 days at between 35.5 to 36.7°C. The solids content was between 5.74 and 7.07%. Biosolids were used in experiments within 24 hours of collection.

CAD2A2D5 (CAD5) cell line

The CAD5 cell line was cloned from highly susceptible cells to PrPSc from the murine catecholaminergic (Cath.a)-differentiated (CAD) cell originated from catecholaminergic neurons in the central nervous system. CAD5 was generously donated by Sukhvir P. Mahal (in the
laboratory of Charles Weissmann, Chair of the Scripps Florida Department of Infectology). The cells were only passed 6-7 times and then reinitiated from fresh stock.

**Mouse-adapted Rocky Mountain Laboratory (RML)-infected mouse brain**

The RML-infected mouse brain is a scrapie-infected brain from the CD1 mouse strain. Mouse brain was homogenized at 10% w/v with phosphate buffer saline (pH 7.40) on ice. It was passed through syringes of decreasing sizes from 18, 20, 22, 24 and 28 gauges. The mouse brain homogenate was aliquoted into 500µL volumes and frozen at -80 °C. The mouse brain was a gift from Sukhvir P. Mahal.

**ELISPOT**

The enzyme-linked immunosorbent spot (ELISPOT) assay was developed by Cecil Czerkinsky in 1983. The ELISPOT assay is a modified version of the ELISA immunoassay. ELISPOT increases the sensitivity of the assay by producing targets as countable spots (Czerkinsky et. al., 1983). A sample of known antigen including proteins, peptides, or glycoproteins is applied to 96 well trays to visualized. In the current research, the antigen was prions infecting CAD5 cells.

**CAD5 cell preparation**

One vial of CAD5 cell was removed from liquid nitrogen long-term storage before starting each assay. Approximately 2.0x10^6 cells (Passage 3) in 1.8 mL of Opti-MEM, 9.1% bovine growth serum (OBGS) medium with 1% penicillin G (10,000 units/mL), streptomycin G (10 mg/mL), and 0.85% saline (Invitrogen, Carlsbad, CA) were used. Cells were centrifuged (500xg for five minutes at room temperature) to remove dimethylsulfoxide (DMSO), and resuspended in 20 mL OBGS medium. The cells were then added to 75 cm² cell culture flasks (1:8 medium per flask) and the flasks were incubated at 37°C in an atmosphere of 5.0% CO₂. The CAD5 cells required three days of incubation to reach 90% confluence and were subcultured when they reached approximately 90 to 95% confluence.

**Quantitative Analysis of Prions**

Scrapie prion proteins were detected by ELISPOT assay (Mahal et. al. 2008). All solutions and reagents used in this assay and vendor information are listed in Table 1.
### Table 1 Solutions and Reagents for ELISPOT Assay

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Recipe and Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opti-MEM, 9.1% bovine growth serum (OBGS)</td>
<td>Opti-MEM (Invitrogen, Carlsbad, CA), 9.1% bovine growth serum (BGS)</td>
</tr>
<tr>
<td></td>
<td>(HyClone Laboratories, Logan, UT), 10,000 units/ml penicillin, and 100µg/ml streptomycin and saline (Invitrogen)</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris-HCl, pH 8.0 (Sigma-Aldrich), 150 mM NaCl, 0.5% Na deoxycholate (Sigma-Aldrich), and 0.5% Trion X-100 (Sigma-Aldrich St. Louis, MO)</td>
</tr>
<tr>
<td>Tris-buffered saline containing 0.1% tween-20</td>
<td>10mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20 (Sigma-Aldrich St. Louis, MO)</td>
</tr>
<tr>
<td>TBST-1% nonfat dry milk</td>
<td>nonfat milk powder (Bio-Rad, Hercules, CA) in 1x TBST</td>
</tr>
<tr>
<td>Guanidinium thiocyanate (GSCN)</td>
<td>10 mM Tris-HCl, pH 8.0, guanidinium thiocyanate (ISC BioExpress, Kaysville, UT)</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF)</td>
<td>Phosphate buffered saline (Fisher Scientific), 2 mM PMSF (Sigma-Aldrich)</td>
</tr>
<tr>
<td>Superblock</td>
<td>1 pouch Superblock dry blend blocking buffer in TBS (Pierce, Rockford, IL)</td>
</tr>
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### Antibodies

- Murine monoclonal antibody ICSM 18 (D-Gen Ltd., London, UK)
- Alkaline phosphatase (AP)-conjugated secondary Goat anti-Mouse IgG1-AP antibody (Southern Biotechnology Associates, Birmingham, AL)

### Substrate

- AP Conjugate Substrate kit (Cat. #170-6432) (Bio-Rad, Hercules, CA)

ICSM 18: Purified mouse immunoglobulin. The antibody isotype is IgG1κ
ELISPOT Assay

Figure 1 illustrates the various steps of the ELISPOT assay.

Exposure of CAD5 Cells to Prion-Infected Samples

Ten-fold serial dilutions of each sample were made in OBGS medium and then 200 µL of each dilution was added to wells of a 96-well cell culture plate (step 1). Approximately 5,000 CAD5 cells were resuspended in 90 µL of OBGS medium and added to the wells (step 2). After four days of incubation at 37°C with 5.0% CO₂ (step 3), the confluent monolayer was suspended by gently pipetting up and down using an 8-channel micropipette. Approximately 20,000 infected CAD5 cells were transferred into each well of the ELISPOT plate (step 4).

Inoculation of ELISPOT Plates

The membrane from the ELISPOT plate was pre-wetted by placing 60 µL of 70% ethanol in each well for two minutes to activate the hydrophobic polyvinylidene fluoride (PVDF) membrane which does not absorb water. The plate was then washed twice with phosphate buffered saline (PBS) (pH 7.40). Approximately 20,000 exposed CAD5 cells to prion-infected samples from the previous step were then transferred to each well of the ELISPOT 96-well plate (step 5). The number of cells used was based on the number of cells in the negative control well. CAD5 cells exposed to a known volume of the brain homogenate were included in the ELISPOT assays as a positive control. The solution was removed from the plate by the vacuum manifold (Pall Corporation, East Hills, NY). The plate was then incubated for one hour at 50°C to allow for the cells to adhere to the plate. Following this incubation period, 50 µL of Proteinase K (30 Hb milliunits/mL, (Roche Diagnostics, Indianapolis, IN) was added to each well and the plate was incubated for an additional 90 minutes at 37°C (step 6). This solution was then removed by the vacuum manifold and washed twice by ImmunoWash MODEL 1575 (Bio Rad, Hercules, CA) with 150 µL of PBS per well. The PBS (pH 7.4) was vacuumed off (step 7) and 150 µL of a 2 mM phenylmethylsulfonyl fluoride (PMSF) was placed in the wells and incubated for 10 minutes at room temperature (~24°C) with constant gentle agitation (step 8). The plate was washed with PBS, and the PBS (pH 7.40) was then vacuumed off. Guanidinium thiocyanate (GSCN) (120 µL of 3 M) was added to each well and the plate was incubated for 10 minutes with constant gentle agitation (step 9). The plate was washed four times by adding and removing 150 µL of PBS to each well. The PBS was vacuumed off the washing steps. After this step, 160 µL of Superblock (Pierce Chemical, Rockford, IL) was added to each well and the plate was incubated at room temperature for one hour with constant gentle agitation (step 10). The Superblock was vacuumed off at the end of the incubation.

Addition of Primary PrP Antibody

A volume of 60 µL of anti-PrP antibody ICSM18 (0.6 µg/mL, D-Gen Ltd., London, UK) was added to each well and the plate was again incubated at room temperature with gentle agitation for one hour (step 11). This primary antibody solution was then removed and the wells were washed four times with 150 µL of tris-buffered saline containing 0.1% tween-20 (TBST) (Sigma-Aldrich, St. Louis, MO). The TBST was vacuumed off at the end of washing.
Addition of Secondary Antibody

A volume of 60 µL of anti-goat IgG-AP antibody (1:5,000 cat. No. 9042-04, Southern Biotechnology Associates, Birmingham, AL) in TBST was added to each well and the plate was incubated at room temperature with gentle agitation for one hour (step 12). The secondary antibody solution was removed and the wells were washed four times with 150 µL of TBST. The ELISPOT membrane was blotted gently on a lint-free tissue and the plate was tapped upside down to remove excess liquid (step 13). The plate was then allowed to air dry at room temperature.

Addition of Enzyme

Next, 60 µL of the alkaline phosphatase reagent (AP 25x color development buffer) was then added to each well and the plate was incubated for 16 minutes at room temperature (step 14). The plate was washed twice with 150 µL of filtered deionized water and the plate tapped upside down to remove any liquid. The plate was allowed to dry inverted at room temperature overnight. Colored spots were observed and the numbers of wells with spots were counted per each dilution. The plate was then stored at -20°C in the dark due to light sensitivity of the spots.

The intensity and size of the spots are different due to variable accumulation of PrP\textsuperscript{Sc} in the individual cells (Mahal et. al., 2008). The proportion of PrP\textsuperscript{Sc}-positive cells is related to the prion concentration. The CAD5 cell line is capable of quantifying RML prion concentration as low as the concentration that can be determined in the mouse bioassay (a standard error of ±20-30%) (Mahal et. al., 2008).
Figure 1 Diagram of ELISPOT assay

*Exposure of CAD5 Cells to Prion-Infected Samples*

1. CAD5 cell line
   The scale represents 100µm

2. 96-well plate

3. ELISPOT plate
Inoculation of ELISPOT Plates

1. Washing and vacuuming is performed after the treatment.
2. Proteinase K denatures PrP and part of PrP\textsuperscript{Sc}.
3. 2 mM PMSF stops Proteinase K reaction.
4. 3M GSCN denatures cells and renders them immunoreactive.
5. Superblock blocks background noise from non-relevant protein.
6. An ELISPOT well contains Prion-infected cells.
7. Proteinase K is applied.
8. Vacuuming is performed after the treatment.
9. Proteinase K denatures PrP and part of PrP\textsuperscript{Sc}.
10. Superblock blocks background noise from non-relevant protein.

A 0.45\textmu m membrane
Addition of Primary PrP Antibody

Addition of Secondary Antibody

Addition of Enzyme

Antibody-reactive spots are diagnostic for scrapie-infected cells
*: Washing and vacuuming is performed after the treatment

**Effect of filtration of prions removal**

To assess the filter pore size to remove the suspended matter and bacteria without the loss of prions from the biosolid eluates, filtration experiments first were performed. Ten microliters of 10% brain homogenate (BH) was added to three vials of 1 mL OBGS medium, and vortexed to distribute the BH evenly in solution. Each vial was filtered through membranes with different pore sizes: 5 µm 29 mm dia. polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA), 0.45 µm with 25 mm cellulose acetate (VWR, West Chester, PA), and 0.22 µm dia 33 mm polyethersulfone membrane (PES) (Millipore, Billerica, MA). PVDF and PES are for low protein binding applications. They were pretreated by 1.5% beef extract (pH 7.20). Ten-fold serial dilutions were performed for each sample. Samples were analyzed by ELISPOT assay.

**Extraction of Prion Proteins from Biosolids**

**Assessment of Phosphate Buffer Saline (PBS)**

Ten microliters of 10% brain homogenate suspended in PBS (pH 7.40), re-homogenized by passage through a 28-gauge needle was added to 1g of Class B biosolids which were previously treated to reduce bacterial numbers by placing in a water bath for 15 minutes at 50°C. It was mixed well by vortexing and centrifuged at 15,000xg for 10 minutes at room temperature.
The supernatant was removed and the sediment fraction was resuspended in 1mL of PBS (pH 7.40) containing 10% penicillin/streptomycin [(penicillin G (10,000 units/mL), streptomycin G (10mg/mL)), and 0.85% saline. The sample was mixed again by vortexing for 10 minutes at room temperature and centrifuged at 15,000 x g for 10 minutes at room temperature followed by aspiration of the supernatant. The supernatant was filtered through a 5 µm pore size filter before assay. The concentration of prions added to the biosolids was assayed by placing 10 µL of 10% brain homogenate in 1mL of PBS containing 10% of penicillin/streptomycin [(penicillin G (10,000 units/mL), streptomycin G (10mg/mL)), and 0.85% saline. The sample was filtered with 5 µm pore size filter 29 mm dia. polyvinylidene fluoride (PVDF) membrane as well. After the filtration, the sample was analyzed by the ELISPOT assay.

**Assessment of 3% Beef Extract**

A method based on the use of beef extract to elute virus from surfaces was developed by Gerba and Goyal (1982) was first tested. To assess the effect of beef extract on the recovery of prions, 10 µL of 10% brain homogenate in PBS was re-homogenized by passage through a 28-gauge needle and added to 1 g of biosolids which were previously treated to reduce bacterial numbers by placing in a water bath for 15 minutes at 50°C. It was mixed well by vortexing and centrifuged at 15,000 x g for seven minutes. The supernatant was removed and the sediment fraction was resuspended in 1 mL of 3% beef extract (pH 7.20) (Becton, Dickinson and Company, Sparks, MD). The sample was mixed by vortexing for 10 minutes at room temperature. It was then centrifuged at 15,000 x g for 10 minutes at room temperature and the supernatant aspirated. The supernatant was filtered through a 5 µm pore size filter. The concentration of prions added to the biosolids was analyzed by adding 10 µL of 10% brain homogenate in 1 mL of PBS containing 10% of penicillin/streptomycin [(penicillin G (10,000 units/mL), streptomycin G (10mg/mL)), and 0.85% saline. The sample was then filtered with a 5 µm pore size filter with 29 mm polyvinylidene fluoride (PVDF) membrane as well. After the filtration, the sample was analyzed by the ELISPOT assay.

An Amicon Ultra-15 centrifugal filter device 10 kDa (Millipore, Billerica, MA) was used to remove the 3% beef extract from the biosolids eluate. Tryptic soy broth (TSB) (1 mL) (Hardy Diagnostic, Santa Maria, CA) was added to the Amicon filter and centrifuged at 3,500 x g for five minutes at room temperature. Any remaining TSB on the filter was discarded and the supernatant added to the Amicon centrifugal device, which was centrifuged at 3,500 x g for 15 minutes at room temperature. OBGS medium (1.0 mL) containing 10% of penicillin/streptomycin [penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline was added to the filter device. The centrifugation step was repeated one additional time. OBGS containing 10% penicillin/streptomycin [penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline was added to bring up the total volume to 1 mL. Samples were then analyzed by ELISPOT.

**Assessment of Sodium Dodecyl Sulfate (SDS)**

Because of the potential toxicity of sodium dodecyl sulfate to cell culture, the effect of various concentration of SDS on the cells was first assessed. To do the assessment, 1 mL of 10, 1, 0.1, 0.01 and 0.001% w/v of sodium dodecyl sulfate (SDS) (Pierce Chemical, Rockford, IL)
were inoculated onto confluent CAD5 cells in a 24-well plate. The plates were observed for four days for the presence of toxicity. Toxicity was determined by observation of cell death.

To assess the ability of SDS to recover prions from biosolids, 10 µL of 10% brain homogenate was re-homogenized by passing through a 28-gauge needle and was then added to 1g of biosolids which were previously treated to reduce bacterial numbers by placing in a water bath for 15 minutes at 50°C. It was thoroughly mixed and centrifuged at 15,000 x g for seven minutes at room temperature. The supernatant was removed and the sediment fraction resuspended in 1 mL of 1% w/v of sodium dodecyl sulfate. The sample was mixed by vortexing for 10 minutes at room temperature. It was then centrifuged at 15,000 x g for seven minutes at room temperature. The supernatant was aspirated and filtered through 5 µm pore size filter pore size 29 mm dia. polyvinylidene fluoride membrane (PVDF) (Millipore, Billerica, MA). The concentration of prions added to the biosolids was analyzed by adding 10 µL of 10% brain homogenate in 1 mL of OBGS containing 10% of penicillin/streptomycin [penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline. The sample was filtered with a 5 µm pore size filter with 29 mm dia. polyvinylidene fluoride (PVDF) membrane as well. After the filtration, the sample was analyzed by the ELISPOT assay.

Removal of SDS

An Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA) with a 10 kDa filter was used to reduce the SDS concentration in the supernatant. Tryptic soy broth (1mL) (TSB) was added to the Amicon device and centrifuged at 3,500 x g for five minutes at room temperature. Any remaining TSB on the filter was discarded and the supernatant added to the Amicon device. It was centrifuged at 3,500 x g for 15 minutes at room temperature. OBGS medium (1.0 mL) containing 10% of penicillin/streptomycin [(penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline (Invitrogen, Carlsbad, CA) was added to the filter device. The centrifugation step was repeated an additional time. OBGS medium containing 10% of penicillin/streptomycin [penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline was added to bring up the total volume to 1 mL. Samples were assayed by ELISpot. In another experimental centrifugation was repeated four additional times.

Assessment of 8 M urea

To assess the toxicity of urea of the CAD5 cells used for the prion assays, 200 µL of 4, 2, 0.4, 0.2, 0.04, 0.02, 0.004 and 0.002 M urea (Sigma-Aldrich, St. Louis, MO) were inoculated with 5,000 cells/ 90 µL of OBGS in a 96-well plate. The plate was incubated for four days to observe toxicity caused by urea. Toxicity was determined of cell death and destructions of the cell monolayer.

To assess the use of 8 M urea for the recovery of prions from biosolids, 20 µL of a 10% brain homogenate suspended in PBS was re-homogenized by passage through a 28-gauge needle and added into two vials containing 1 g of biosolids which were previously treated to reduce bacteria numbers by placing in a water bath for 15 minutes at 50°C. They were mixed well by vortexing and centrifuged at 8,000 x g for seven minutes at room temperature. The supernatant was removed and the sediment fraction was resuspended in 1 mL of 8 M urea (pH 7.53)
(Sigma-Aldrich, St. Louis, MO). One sample was mixed by vortexing for 10 minutes at room temperature and the other heated at 80°C for 10 minutes without vortexing. They were centrifuged at 8,000 xg for seven minutes at room temperature and the supernatants were aspirated. The supernatants were then filtered using a 5 µm pore size filter 29 mm dia. polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The concentration of prions added to biosolids was analyzed by placing 20 µL of 10% brain homogenate to 1 mL of OBGS containing 10% of penicillin/streptomycin (penicillin G (10,000 units/mL), streptomycin G (10 mg/mL), and 0.85% saline. The sample was filtered with 5 µm pore size filter 29 mm dia. PVDF membrane as well. After the filtration, the sample was analyzed by the ELISPOT assay.

Removal of Urea from Supernatants

An Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA) with a 10 kDa filter was used to reduce the concentration of the 8 M urea (pH 7.53) from the supernatants. Tryptic soy broth (1mL) (TSB) was added to the Amicon device and centrifuged at 3,500 xg for five minutes at room temperature. Any residue of TSB on the filter was discarded and the supernatant added to the Amicon device which was centrifuged at 3,500 xg for 15 minutes at room temperature. OBGS medium (0.5 mL) containing 10% of [penicillin G (10,000 units/mL), streptomycin G (10 mg/mL)], and 0.85% saline was added to the filter device. The centrifugation step was repeated two more times. OBGS medium containing 10% of penicillin G (10,000 units/mL), streptomycin G (10 mg/mL), and 0.85% saline (Invitrogen, Carlsbad, CA) was added to adjust the total volume to 1 mL. Samples were assayed by ELISPOT.

Assessment of PBS (pH 7.4) heating at 80°C

To assess the use of phosphate buffered saline (pH 7.4) heating at 80°C for the recovery of prions from biosolids, 20 µL of a 10% brain homogenate suspended in PBS was re-homogenized by passage through a 28-gauge needle and added into two vials containing 1 g of biosolids which were previously treated to reduce bacteria numbers by placing in a water bath for 15 minutes at 50°C. They were mixed well by vortexing and centrifuged at 8,000 xg for seven minutes at room temperature. The supernatant was removed and the sediment fraction was resuspended in 1 mL of PBS (pH 7.40). The sample was vortexed and then heated at 80°C for 10 minutes without agitation in the oven. It was mixed again shortly after heating by vortexing. They were then centrifuged at 8,000 xg for seven minutes at room temperature and the supernatants were aspirated. The supernatants were then filtered using a 5 µm pore size filter 29 mm dia. polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The concentration of prions added to biosolids was analyzed by placing 20 µL of 10% brain homogenate to 1 mL of PBS containing 10% of penicillin/streptomycin (penicillin G (10,000 units/mL), streptomycin G (10 mg/mL), and 0.85% saline. The sample was filtered with 5 µm pore size filter 29 mm dia. PVDF membrane as well. After the filtration, the sample was analyzed by the ELISPOT assay.
**Survival of Prion Proteins in Class B Biosolids at Mesophilic and Thermophilic Temperatures**

**Sample Preparation**

To assess the persistence of infectivity during anaerobic digestion temperatures, 20 µL of a 10% brain homogenate in PBS was re-homogenized by passage through a 28-gauge needle and added to 1.5 mL safe-lock tubes manufactured by polypropylene (Eppendorf, Westbury, NY) containing 1g of Class B biosolid which were previously treated to reduce bacterial numbers by placing in a water bath for 15 minutes at 50°C. They were mixed for ten minutes, and a sample for assay taken immediately. The samples held by mesophilic temperature conditions were incubated at 37°C for 15 days in an incubator. To simulate thermophilic conditions, samples were incubated at 60°C for 10 days in an oven. To assay concentration of prions in PBS, 60 µL of 10% brain homogenate into 3 mL of PBS containing 10% of penicillin/streptomycin [penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline. It was vortexed well and was distributed to 1 mL PBS for each test condition (none, mesophilic and thermophilic). They were filtered with a 5 µm pore size filter 29 mm dia. polyvinylidene fluoride (PVDF) membrane. Class B biosolids (1 g) were heated for 15 minutes at 50°C in a water bath without brain homogenate and also assayed on cell culture as negative control.

**Sample Processing**

To recover the prions, the biosolid samples were centrifuged at 8,000 x g for seven minutes at room temperature. The samples containing PBS were not centrifuged. The supernatant was removed and the sediment fraction was resuspended in 1 mL of 4 M urea (pH 7.33). Vials were vortexed and then heated at 80°C for 10 minutes without agitation in the oven. Samples were mixed again shortly after heating by vortexing. They were centrifuged at 8,000 x g for seven minutes at room temperature and the supernatants were aspirated. The supernatants were then filtered through a 5 µm pore size filter 29 mm dia. polyvinylidene fluoride (PVDF) membrane.

**Removal of 4 M Urea from Supernatants**

An Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA) with a 10 kDa filter was used to remove the 4 M urea (pH 7.33) from the supernatants. Tryptic soy broth (TSB) (1 mL) was added to the Amicon device and centrifuged at 3,500 x g for ten minutes at room temperature. Any remaining TSB on the filter was discarded and the supernatant added to the Amicon device which was centrifuged at 3,500 x g for 15 minutes. PBS (1 mL) (pH 7.40) containing 10% of [penicillin G (10,000 units/mL), streptomycin G (10mg/mL)], and 0.85% saline was added to the supernatant added to the filter device. The centrifugation step was repeated two more times. PBS containing 10% penicillin G (10,000 units/mL), streptomycin G (10mg/mL), and 0.85% saline was added to adjust the total volume to 1 mL. Samples were then assayed by ELISPOT.
Data Analysis

Photographs of the ELISPOT assay were done via a microscope, the Leica EZ4 digital educational stereomicroscope with a 4.4:1 zoom having high quality imaging system (Leica Microsystems, Switzerland), supplied with Leica LAS EZ software. The zoom magnification from 8X – 35X provides high-resolution images. PrPSc infected cells appear as dark purple spots on the membrane of the ELISPOT plate (Figures 2, 4 and 6). Wells showing growth of prions developed dark spots. Some spot development on non-infected cells occur as background. Usually the non-infected cells background contained one to five spots while infected cells contained more than ten spots per well.

The tissue culture infectious dose 50% technique (TCID_{50}) was used to calculate the titer of PrPSc. TCID_{50} is the indirect method to measure the dilution to achieve 50% infection of the tested units. The titer of the sample was calculated by the following Reed-Muench formula as the infectivity titer:

\[
\text{TCID}_{50} = \text{Dilution where spots > 50\%} + \left[ \frac{\% \text{ positive above 50\%} - 50\%}{\% \text{ positive above 50\%} - \% \text{ positive below 50\%}} \right] \times \log_{10}10
\]

The minimum detection limit for this study was 3.16 TCID_{50} per 200 µL. It was determined via the following calculation:

\[
10^0 + \left[ \frac{100-50}{100-0} \right] \times \log_{10}10 = 10^{0.5} = 3.16 \text{ TCID}_{50}
\]

The titer calculated by TCID_{50} was multiplied by the total volume in each well (290 µL) divided by the volume containing 20,000 infected cells (for the test samples) or uninfected cells (for the negative control) to calculate the titer in each well. In order to calculate the total concentration of PrPSc per each sample (1 mL), this was multiplied by 1 mL divided by each dilution (200 µL) added to a well of a 96-well cell culture plate.

\[
\text{Total Conc.}(\text{TCID}_{50}/\text{total}) = \text{TCID}_{50} \times \frac{290\mu L}{\text{Volume containing 20,000 cells}} \times \frac{1\text{mL}}{200\mu L}
\]
Figure 2. Picture of an ELISPOT well at $10^{-3}$ dilution infected with $10^6$ prions

Figure 3. Picture of an ELISPOT well at $10^{-3}$ dilution infected with $10^6$ prions (inverted color)
Figure 4. Picture of an ELISPOT well at $10^{-4}$ dilution infected with $10^6$ prions

Figure 5. Picture of an ELISPOT well at $10^{-4}$ dilution infected with $10^6$ prions (inverted color)
Figure 6. Picture of an ELISPOT well of uninfected cells (negative control)

Figure 7. Picture of an ELISPOT well of uninfected cells (negative control) (inverted color)
RESULTS

Effect of filtration on loss of prions

A series of filtration experiments was assessed to determine if suspended matter and bacteria in the assay could be reduced without significant loss of prions (Table 2). Filtration experiments with 10 μL of 10% brain homogenate w/v were conducted with different pore sizes of filters including 5 μm, 0.45 μm and 0.22 μm. PrP\(^{Sc}\) was reduced by 68.4% after filtration through a 5 μm filter. This increased to 96.8% after passage through a 0.45 μm pore size filter. Filtration through a 0.22 μm pore size removed 98.2%. The removal of the prions by the membrane filters was probably due to the association of the prions with cell debris in the brain homogenate. It was concluded the filtration of the biosolid eluates to remove bacteria with 0.45 μm and 0.22 μm filters was not feasible. Therefore samples were passed through a 5 μm filter and heat treated at 50°C for 15 minutes to reduce bacterial numbers before cell culture assay.

Recovery of prions from biosolids

Subsequent to this, a series of experiments were performed to develop a method for the recovery of prions from biosolids. Phosphate buffered saline, sodium dodecyl sulfate (SDS), beef extract, urea and urea heating at 80°C were assessed. Table 3 shows recovery of prion proteins from biosolids with phosphate buffered saline (PBS). While the recovery of prions was only 0.01%, no toxicity of the extract to the cell culture was observed. Beef extract is a protein solution which is commonly used to recover viruses adsorbed to surfaces. Recovery of prion proteins from biosolids with 3% beef extract was less than 0.001%, but no toxicity to the cell culture was observed (Table 4).

Table 2. Effect of filtration on removal of prions from brain homogenates

<table>
<thead>
<tr>
<th>Filter pore size (um)</th>
<th>TCID(_{50})/total</th>
<th>% Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before filtration</td>
<td>1.78E+04</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>5.62E+03</td>
<td>68.4</td>
</tr>
<tr>
<td>0.45</td>
<td>5.62E+02</td>
<td>96.8</td>
</tr>
<tr>
<td>0.22</td>
<td>3.16E+02</td>
<td>98.2</td>
</tr>
</tbody>
</table>

% Removed = 100% - (Spots/total / Before filtration * 100%)
Table 3. Recovery of prions from biosolids with phosphate buffered saline (PBS) (0.01M) (pH 7.40)

<table>
<thead>
<tr>
<th>% Total Dry Solids</th>
<th>Conc. prions added to biosolids (TCID$_{50}$/total)</th>
<th>Conc. of prions recovered (TCID$_{50}$/total)</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.07</td>
<td>1.00E+06</td>
<td>100</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Conc.: Concentrate
% Conc. Recovered = Conc. of prions recovered / Conc. prions added to biosolids * 100%
Weight of sample processed: 1.16g
No toxicity

Table 4. Recovery of prions from biosolids with 3% beef extract (pH 7.20)

<table>
<thead>
<tr>
<th>% Total Dry Solids</th>
<th>Conc. prions added to biosolids (TCID$_{50}$/total)</th>
<th>Conc. of prions recovered (TCID$_{50}$/total)</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.07</td>
<td>2.87E+05</td>
<td>&lt;3.16</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Conc.: Concentrate
% Conc. Recovered = Conc. of prions recovered / Conc. prions added to biosolids * 100%
Weight of sample processed: 1.03g
No toxicity
<3.16 per 200µL
SDS is a strong detergent that has been used to recover prions from anaerobically digested sludge (Glen et al. 2008). However, as shown in Table 5, it is very toxic to cell culture. Instant cell death was observed immediately after SDS was inoculated on CAD 5 cells at concentrations of 10, 1, and 0.1% (w/v). Toxicity was evident for 0.01% SDS after one day. No toxicity was observed for 0.001% SDS after four days.

Centrifugal filter devices are designed to concentrate low molecular weight compounds such as proteins and nucleic acids, as well as microorganisms. In order to remove 1% SDS from biosolid eluates, the Amicon Ultra-15 centrifugal filter device (Table 6) was used. Using this method, 32% of PrP$_{Sc}$ was recovered after the sample was centrifuged twice to eliminate SDS. Recovery of PrP$_{Sc}$ from another sample which was centrifuged four times to eliminate SDS decreased to 4.7%. Toxicity was observed in the $10^{-1}$ and $10^{-2}$ dilutions after 4 days of incubation for both samples. Additional centrifugation treatments did not reduce toxicity in cell culture, but did result in a further reduced recovery efficiency.

Urea is non-ionic and chaotropic, and was tested for its ability to recover PrP$_{Sc}$ from biosolids. Table 7 shows the results of toxicity tests for various concentrations of urea on CAD5 cells. Concentrations of urea greater than 0.4 M caused toxicity after four days of incubation. Concentrations lower than 0.2 M of urea were not toxic for CAD5 cells.
Table 5. Assessment of sodium dodecyl sulfate (SDS) toxicity on CAD5 cells

<table>
<thead>
<tr>
<th>Concentration of SDS (% w/v)</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Toxic after 1 minute</td>
</tr>
<tr>
<td>1</td>
<td>Toxic after 1 minute</td>
</tr>
<tr>
<td>0.1</td>
<td>Toxic after 1 minute</td>
</tr>
<tr>
<td>0.01</td>
<td>Toxic after 1 day</td>
</tr>
<tr>
<td>0.001</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Negative: no toxicity

Table 6. Extraction of prion proteins from biosolids with sodium dodecyl sulfate (SDS) effect of membrane centrifugation

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Total Dry Solids</th>
<th>Weight of Sample Processed (g)</th>
<th>Dilution, at which toxicity observed</th>
<th>Conc. prions added to biosolids (TCID₅₀/total)</th>
<th>Conc. of prions recovered (TCID₅₀/total)</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.07</td>
<td>1.09</td>
<td>10⁻¹ and 10⁻²</td>
<td>2.87E+05</td>
<td>9.10E+04</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>5.74</td>
<td>1.05</td>
<td>10⁻¹ and 10⁻²</td>
<td>3.95E+05</td>
<td>1.85E+04</td>
<td>4.7</td>
</tr>
</tbody>
</table>

% Conc. Recovered = Conc. of prions recovered / Conc. prions added to biosolids * 100%

Sample 1: Two times of centrifugation
Sample 2: Four times of centrifugation
Table 7. Assessment of urea toxicity on CAD5 cells
Negative: no toxicity

<table>
<thead>
<tr>
<th>Concentration of Urea (M)</th>
<th>Toxicity (After 4 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>Toxic</td>
</tr>
<tr>
<td>2.0</td>
<td>Toxic</td>
</tr>
<tr>
<td>0.4</td>
<td>Toxic</td>
</tr>
<tr>
<td>0.2</td>
<td>Negative</td>
</tr>
<tr>
<td>0.04</td>
<td>Negative</td>
</tr>
<tr>
<td>0.02</td>
<td>Negative</td>
</tr>
<tr>
<td>0.004</td>
<td>Negative</td>
</tr>
<tr>
<td>0.002</td>
<td>Negative</td>
</tr>
</tbody>
</table>

When urea (8 M) was used as the eluate it was subsequently centrifuged in the Amicon filter to lower the concentration of urea to a non-toxic concentration. Table 8 represents the results of prion protein extraction using 8 M urea at two different temperatures. One sample was exposed to 80°C for 10 minutes and the other was agitated by slow vortexing for 10 minutes at room temperature to compare the recovery of PrP<sup>Sc</sup>. PrP<sup>Sc</sup> was recovered with an efficiency of 31.7% from the biosolids when it was heated to 80°C for 10 minutes. On the other hand, less than 0.002% of PrP<sup>Sc</sup> was recovered from biosolids in urea at room temperature. Toxicity was observed at a 10<sup>-1</sup> dilution of the eluate, but not at greater dilutions. The results demonstrate that heating the extraction solution improved the percent recovery of prion proteins from biosolids probably because of denaturization of the prion proteins. Since infectious prions were detected following the extraction, denatured proteins must have re-annealed at cooler temperatures.

Elution of prions in phosphate buffered saline was also evaluated at 80°C. Since phosphate buffered saline (pH 7.4) did not cause toxicity for the CAD5 cell line, the centrifugal filter step was not performed to assess the recovery of prions with PBS at 80°C. Here the percent recovered was 0.032% (Table 9). Compared to the efficiency of 31.7% with 8 M urea heating at 80°C, the recovery was much lower. Apparently PBS was not efficient for the extraction of prions from biosolids.
Table 8. Recovery of prions with 8 M urea from biosolids at 80°C and at room temperature

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight of Sample Processed (g)</th>
<th>Toxicity (dilution)</th>
<th>Conc. prions added to biosolids (TCID₅₀/total)</th>
<th>Conc. of prions recovered (TCID₅₀/total)</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>80°C</td>
<td>1.05</td>
<td>10⁻¹</td>
<td>1.80E+05</td>
<td>5.70E+04</td>
<td>31.7</td>
</tr>
<tr>
<td>Room Temperature</td>
<td>1.02</td>
<td>10⁻¹</td>
<td>1.80E+05</td>
<td>&lt;3.16</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

% Conc. Recovered = Conc. of prions recovered / Conc. prions added to biosolids * 100%
Total Dry Solids: 5.74%
<3.16 per 200µL

Table 9. Recovery of prions with PBS (pH 7.40) from biosolids at 80°C

<table>
<thead>
<tr>
<th>% Total Dry Solids</th>
<th>Weight of Sample Processed (g)</th>
<th>Conc. prions added to biosolids (TCID₅₀/total)</th>
<th>Conc. of prions recovered (TCID₅₀/total)</th>
<th>% Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.85</td>
<td>1.02</td>
<td>9.06E+05</td>
<td>2.86E+02</td>
<td>0.032</td>
</tr>
</tbody>
</table>

No toxicity was observed in the sample

From these experiments it was decided that treatment of the biosolids with 4 M urea at 80°C for ten minutes was the best option for recovery of the infectious prions from the biosolids, with the least amount of toxicity to the cells used in the assay.

To assess the persistence of infectivity at anaerobic digestion temperatures, prions were added to mesophilic (37°C) anaerobic digested biosolids and held at temperatures characteristic of either a mesophilic and thermophilic (60°C) digester. Table 10 shows the recovery of prion proteins with 4 M urea from biosolids with 80°C treatment followed by treatment with the Amicon filter to reduce toxicity. Prion recovery was 17.2% from the biosolids and no toxicity was observed after four days of incubation. No indigenous prions were detected in the biosolids used in this experiment as shown by the assay of biosolids without extreme prion addition. Table 11 shows the reduction of prion proteins in biosolids incubated at mesophilic or thermophilic temperatures for 15 days. Control samples without biosolids were conducted on PBS at both temperatures.
Table 12 shows the log\textsubscript{10} reduction of infectious prions after holding at mesophilic and thermophilic temperatures in biosolids. A 2.43-log\textsubscript{10} reduction was observed under mesophilic temperature conditions, and a 3.41-log\textsubscript{10} reduction under thermophilic temperature conditions. The greatest reduction was observed after 10 days at 60 °C (Figure 8).

A 1.13-log\textsubscript{10} reduction was observed under mesophilic temperature conditions, and a 1.80-log\textsubscript{10} reduction was observed under thermophilic temperature conditions in PBS (Table 13). The greatest reduction was observed after 10 days at 60°C. Thus a greater decline in prion infectivity occurred in the biosolids held at the same temperature than in the PBS.
Table 10. Recovery of prions with 4 M urea from biosolids at 80°C

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight of Sample Processed (g)</th>
<th>Conc. prions added to biosolids (TCID$_{50}$/total)</th>
<th>Conc. of prions recovered (TCID$_{50}$/total)</th>
<th>% Recovered</th>
<th>Average % Recovered</th>
<th>Standard Deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.03</td>
<td>9.06.E+05</td>
<td>2.87.E+05</td>
<td>31.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.99</td>
<td>9.06.E+05</td>
<td>9.06.E+04</td>
<td>10.0</td>
<td>17.2</td>
<td>12.5</td>
</tr>
<tr>
<td>3</td>
<td>1.01</td>
<td>9.06.E+05</td>
<td>9.06.E+04</td>
<td>10.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No toxicity was observed in any of the samples
Conc.: Concentrate
% Conc. Recovered = Conc. of prions recovered / Conc. prions added to biosolids * 100%
There was no prions detected from the original biosolids
% Total dry solids: 6.85%
Table 11. Reduction of prion proteins after incubation of biosolids at mesophilic and thermophilic digestion temperatures

<table>
<thead>
<tr>
<th>Sample</th>
<th>Test Conditions</th>
<th>Holding Time (days)</th>
<th>Weight of Sample Processed (g)</th>
<th>Control Conc. Of Prions in PBS (TCID_{50}/total)</th>
<th>Conc. of prions recovered from biosolids (TCID_{50}/total)</th>
<th>Average Conc. Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>0</td>
<td>1.03</td>
<td>2.87.E+05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>0</td>
<td>9.06.E+05</td>
<td>9.06.E+04</td>
<td>9.06.E+04</td>
<td>1.56.E+05</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>0</td>
<td>1.01</td>
<td>9.06.E+04</td>
<td>9.06.E+04</td>
<td>1.56.E+05</td>
</tr>
<tr>
<td>1</td>
<td>Mesophilic</td>
<td>15</td>
<td>1.02</td>
<td>6.79.E+04</td>
<td>1.45.E+02</td>
<td>5.80.E+02</td>
</tr>
<tr>
<td>2</td>
<td>Mesophilic</td>
<td>15</td>
<td>1.01</td>
<td>1.45.E+02</td>
<td>1.45.E+02</td>
<td>5.80.E+02</td>
</tr>
<tr>
<td>3</td>
<td>Mesophilic</td>
<td>15</td>
<td>1.02</td>
<td>1.45.E+02</td>
<td>1.45.E+02</td>
<td>5.80.E+02</td>
</tr>
<tr>
<td>1</td>
<td>Thermophilic</td>
<td>10</td>
<td>1.01</td>
<td>6.79.E+04</td>
<td>1.45.E+02</td>
<td>6.05.E+01</td>
</tr>
<tr>
<td>2</td>
<td>Thermophilic</td>
<td>10</td>
<td>1.02</td>
<td>6.79.E+04</td>
<td>1.45.E+02</td>
<td>6.05.E+01</td>
</tr>
<tr>
<td>3</td>
<td>Thermophilic</td>
<td>10</td>
<td>1.02</td>
<td>6.79.E+04</td>
<td>1.45.E+02</td>
<td>6.05.E+01</td>
</tr>
</tbody>
</table>

Room Temp: ~24°C
Mesophilic: 37°C
Thermophilic: 60°C
Control held in PBS at both mesophilic and thermophilic temperatures
Initial prion concentration added to biosolids at either temperature was 9.06E + 05
Table 12. Log$_{10}$ reduction of infectious prions after holding at meshophilic and thermophilic temperatures in biosolids

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Average $N_0$</th>
<th>Time (days)</th>
<th>$N$</th>
<th>$N/N_0$</th>
<th>$\text{Log}_{10} N/N_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesophilic</td>
<td>1.56.E+05</td>
<td>15</td>
<td>5.80.E+02</td>
<td>3.72.E-03</td>
<td>-2.43</td>
</tr>
<tr>
<td>Thermophilic</td>
<td>10</td>
<td>6.05.E+01</td>
<td>3.88.E-04</td>
<td>-3.41</td>
<td></td>
</tr>
</tbody>
</table>

Mesophilic: 37 °C  
Thermophilic: 60 °C  
$N_0$: Average of conc. recovered at control start from the experiment in Table 10  
$N$: Average of conc. recovered from the experiment in Table 10

Table 13. Log$_{10}$ reduction of infectious prions after holding at meshophilic and thermophilic temperatures in PBS

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Average $N_0$</th>
<th>Time (days)</th>
<th>$N$</th>
<th>$N/N_0$</th>
<th>$\text{Log}_{10} N/N_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesophilic</td>
<td>9.06.E+05</td>
<td>15</td>
<td>6.79.E+04</td>
<td>7.49.E-02</td>
<td>-1.13</td>
</tr>
<tr>
<td>Thermophilic</td>
<td>10</td>
<td>1.45.E+04</td>
<td>1.60.E-02</td>
<td>-1.80</td>
<td>9.06.E+05</td>
</tr>
</tbody>
</table>

Mesophilic: 37 °C  
Thermophilic: 60 °C  
$N_0$: Average of conc. recovered at control start from the experiment in Table 10  
$N$: Average of conc. recovered from the experiment in Table 10
Figure 8. Prion protein reduction after holding in biosolids and PBS at mesophilic and thermophilic temperatures

<table>
<thead>
<tr>
<th>Initial Concentration</th>
<th>Mesophilic</th>
<th>Thermophilic</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCID₅₀/total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>1.E+00</td>
<td>1.E+00</td>
</tr>
<tr>
<td>Biosolids</td>
<td>1.E+00</td>
<td>1.E+00</td>
</tr>
</tbody>
</table>
DISCUSSION

The goal of this project was to develop a method for the recovery of infectious prions from biosolids and then to apply this method to study the infectivity of the prions in biosolids held at temperatures and conditions characteristic of mesophilic and thermophilic anaerobic digestion.

Because the biosolid eluates contain particles and bacteria which interfere with the ELISPOT assay, the effect of filtration on the removal of brain homogenates was first assessed. It was found that membrane filtration with pore sizes of 0.45 and 0.22 µm, reduced the concentration of prions by more than 95%. Prions are smaller than these pore sizes; however they are most likely attached via sorption particles in the brain homogenates. The loss of prions was minimized by the use of the larger sized 5 µm pore size filter. These data also illustrate that prions sorbed to particles are still infectious.

Before survival of prions added to the biosolids could be assessed, they had to be recovered from the biosolids. Thus, several different eluents were assessed for their recovery. Phosphate buffered saline (PBS), 3% beef extract, SDS and urea without and with heat treatment were studied. Beef extract has been commonly used for elution of viruses from biosolids. However, it did not work well for PrP<sup>Sc</sup> (<0.001%). This is likely due to the high affinity between PrP<sup>Sc</sup> and biosolids. PBS was also found to be inefficient for the recovery of the prions (0.01% without heating and 0.032% with heating).

Glen et al. (2008) successfully recovered 71% of PrP<sup>TSE</sup> from anaerobic sludge using 1% sodium dodecyl sulfate (SDS). However, they analyzed the results by immunoblotting with SDS-PAGE sample buffer which is a presence-absence type of assay and does not require the use of cell culture. In our study, the ELISPOT assay was used to analyze the infectivity of prion proteins in the CAD5 cell line. Toxicity was evident in the cell culture when using 0.01% SDS after one day. No toxicity was observed with 0.001% SDS after four days. However, the low concentration of 0.001% SDS was too low a concentration for efficient extraction of the PrP<sup>Sc</sup> from the biosolids. Thus, a centrifugal filter device was tested to reduce the concentration of SDS before cell culture assay. However, not all of the toxicity could be eliminated by this method. It was still necessary to dilute the eluate 10<sup>-1</sup> to 10<sup>-2</sup> before assay. Thus, we were not able to effectively reduce the concentrate total of 1% (w/v) SDS to a level that was not toxic to the cell culture.

The PrP<sup>Sc</sup> molecule consists of both hydrophobic and hydrophilic regions (Cooke et al., 2007) and forms ordered aggregates and readily attaches to components in biosolids. When aggregation of PrP<sup>Sc</sup> occurs, the proteins bind tightly to each other (forming complex tertiary structures). Under alkaline condition (above pH 8), the size of the aggregates decrease (Xin et al., 2007), so the pH is an important factor in the extraction of PrP<sup>Sc</sup> from particles. Class B biosolid samples used in this study were alkaline (pH ~8.5).

Ma et al. (2007) extracted PrP<sup>Sc</sup> from quartz sand using 10 M urea (in 0.01 M Tris HCl, pH 7.4) exposed at 100 °C for 10 minutes. PrP<sup>Sc</sup> was detected by a double-antibody sandwich enzyme-linked immunosorbet assay (ELISA). Samples were diluted to less than 0.5 M urea
with analysis via a colorimetric ELISA. Based on their study, urea served as an effective PrP\textsuperscript{Sc} extraction solution from sand. Additionally, Leita et al. (2006) demonstrated no interference of PrP\textsuperscript{Sc} detection using 4 M urea (in water, pH 7.0) by immunoblotting.

High concentrations of urea (>0.4 M in type II reagent water) cause toxicity for the cell lines used in this study after four days of incubation. Therefore, a centrifugal filter device was used to reduce the original concentration of the urea in the eluate. When 8 M of urea was used, toxicity was still observed in the 10\textsuperscript{-1} dilution of the eluate. The toxicity limited the sensitivity of the assay. The concentration of the urea was reduced to 4 M (held at 80°C for ten minutes, followed by treatment with the centrifugal filter to reduce the urea concentration before assay on the cell culture to reduce cell toxicity. This was found to recover prions from the biosolids with an efficiency of 17.2%.

According to Xin et al. (2007), the size of prion aggregates decreased when the pH increased to 8. The pH of the urea was adjusted to 7.33 to maintain the alkalinity of Class B biosolids (pH ~8.5) in order to limit the size of PrP\textsuperscript{Sc} aggregates. Urea (H\textsubscript{2}NCONH\textsubscript{2}) is both non-ionic and chaotropic and can strongly bind water molecules. As a result, tertiary structures of proteins are disrupted, and the non-covalent bonds such as hydrophobic interactions are effectively disrupted (Gerba 1984). Urea works as a powerful protein denaturant.

Kirchmayr et al. (2006) demonstrated that incubation of PrP\textsuperscript{Sc} under meshophilic conditions (35°C) did not show any significant reduction by western blotting. However, based on the infectivity, we observed a 2.43-log\textsubscript{10} reduction of PrP\textsuperscript{Sc}. Thus, it appears that detection by western blot is not reflective of the loss of infectivity. In contrast, they did observe a reduction of PrP\textsuperscript{Sc} under thermophilic conditions. The PrP\textsuperscript{Sc} titer was reduced about one order of magnitude after the incubation time of 302 hours (12 days) (at 55°C). In our study, the infectivity of PrP\textsuperscript{Sc} was decreased by 3.41-log\textsubscript{10} after an incubation period of 10 days (at 60°C). Thus, infectivity loss is faster than the degradation of the prion protein. The infectivity of the prions was reduced under mesophilic temperatures by 2.43-log\textsubscript{10} and thermophilic by 3.41-log\textsubscript{10}. Thus, there was a greater reduction in infectivity in the biosolids than the PBS (mesophilic temperature by 1.13-log\textsubscript{10} and thermophilic temperature by 1.80-log\textsubscript{10}). The greater inactivation of the prions in the biosolids could be due to the production of proteolytic enzymes or other substances which denature the proteins. It is also possible that ammonia or other substances in biosolids could result in a faster denaturalization of the prions.

The survival of scrapie experiment in raw sewage was studied by Maluquer de Motes et al. (2008). They studied the survival of PrP\textsuperscript{Sc} using the western blotting. They observed a 90% reduction of detectable PrP\textsuperscript{Sc} after 32.6 days of incubation at 20 °C, and a 99% reduction after 51.2 days. In our study, a 99.1% infectivity reduction of PrP\textsuperscript{Sc} infectivity was observed after 15 days at 37°C.
CONCLUSIONS

This was the first study of the survival of infectious prions in biosolids. To accomplish this, a method was developed for extracting prions from biosolids that was compatible with the assay of the samples using cell culture. Heating biosolids samples with 4 M urea and reducing the urea concentration by membrane centrifugation resulted in a method capable of recovering prions with an efficiency of 17.2%.

This method was then used to study the persistence of prion infectivity in Class B biosolids incubated at mesophilic (37°C) and thermophilic (60°C) anaerobic digester temperatures. A 2.43-log₁₀ reduction in prion infectivity was observed under a mesophilic (37°C) temperature after 15 days and 3.41-log₁₀ reduction was observed after 10 days at a thermophilic temperature (60°C). This loss of infectivity was greater than in PBS, suggesting factors other than temperature are responsible for the loss of infectivity of the prions.

A method has been developed that will allow for additional detailed studies on the survival of prions in biosolids.

ACKNOWLEDGEMENTS

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