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Application of protein misfolding cyclic amplification to detection of prions in anaerobic digestate

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ABSTRACT

The exceptional physio-chemical resistance of prions to established decontamination procedures poses a challenge to assessing the suitability of applied inactivation methods. Prion detection is limited by the sensitivity level of Western blotting or by the cost and time factors of bioassays. In addition, prion detection assays can be limited by either the unique or complex nature of matrices associated with environmental samples. To investigate anaerobic digestion (AD) as a practical and economical approach for potential conversion of specified risk materials (SRM) into value added products (i.e., renewable energy), challenges associated with detection of prions in a complex matrix need to be overcome to determine potential inactivation. Protein misfolding cyclic amplification (PMCA) assay, with subsequent Western blot visualization, was used to detect prions within the AD matrix. Anaerobic digestate initially inhibited the PMCA reaction and/or Western blot detection. However, at concentrations of ≤1% of anaerobic digestate, 263 K scrapie prions could be amplified and semi-quantitatively detected. Infectious 263 K prions were also proven to be bioavailable in the presence of high concentrations of digestate (10–90%). Development of the PMCA application to digestate provides extremely valuable insight into the potential degradation and/or fate of prions in complex biological matrices without requiring expensive and time-consuming bioassays.

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1. Introduction

Transmissible spongiform encephalopathies (TSEs) are a group of unique infectious and ultimately fatal neurodegenerative diseases that affect both animals and humans. TSEs include scrapie of sheep and goats, chronic wasting disease (CWD) of cervids, bovine spongiform encephalopathy (BSE) of cattle, and Creutzfeldt–Jakob disease (CJD) in humans. The etiological agent responsible for these diseases is believed to be a self-replicating host protein known as a prion (Prusiner, 1982). The disease process involves the conversion of normal cellular prion protein (PrP^C) present in both animal and human neuronal tissue to a misfolded, protease resistant conformation (PrP^{Sc}) that upon aggregation results in disease (Pan et al., 1993; Yuan et al., 2006). The unique properties of PrP^{Sc} make it extremely resistant to established decontamination procedures such as preservation in fixatives, heat, combustion, ultraviolet light and gamma radiation (Gibbs et al., 1978; Latarjet et al., 1970; Suyama et al., 2007).

Prion detection is complicated due to the fact that both the normal and infectious conformers have the exact same primary amino acid sequence, only differing by protein structure (Oesch et al., 1985). Most immunodetection assays have therefore had to rely on proteinase K (PK) digestion of brain homogenates, which eliminates the PK-sensitive normal prion protein (PrP^C) conformer. After PK digestion, proteins remaining in the brain homogenate can be separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and PK-resistant PrP^{Sc} visualized using non-discriminatory prion protein antibodies. However, given the extremely low concentration of PrP^{Sc} found in tissues and/or fluids outside of the brain of infected animals and the detection limit of standard Western blotting (~10–20 pM; 5–10 pg of PrP) (Ingrosso et al., 2002; Lee et al., 2000), the Western blot technique is often inadequate for detection at low concentrations. To date animal bioassays remain the gold standard for proving infectivity, but are expensive and time-

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consuming. Using bioassay to evaluate prion inactivation technologies should only be undertaken if in vitro assay results look promising.

One such in vitro assay, shown to correlate with animal infectivity in studies examining heat sterilization is protein, misfolding cyclic amplification (PMCA) (Murayama et al., 2006; Suyama et al., 2007). The PMCA assay is based on template-directed misfolding of PrP^{C} with the addition of a PrP^{Sc} (Saa et al., 2006; Saborio et al., 2001). Using PMCA amplification prior to standard Western blotting increases the limit of detection for PrP^{Sc} by several logs of magnitude (dependent on the number of PMCA rounds applied) and can be used to quantify results. The usefulness of the PMCA assay in application to complex matrices has been successfully demonstrated in detection of PrP^{Sc} in both soil and feces (Kruger et al., 2009; Nagaoka et al., 2010). Because the PMCA assay relies on template-directed misfolding of normal PrP^{C} into the infectious PrP^{Sc} form, interferences from background matrix components which prevent protein conversion could result in inaccurate results. In particular, false-negative results may occur if PrP^{Sc} is bound by matrix components (e.g., humic substances, organic matter, minerals) that make it unable to convert PrP^{C}. Alternatively, if PrP^{C} interacts with matrix components, it may be unavailable for conversion to PrP^{Sc}. The exact mechanism of protein conversion from PrP^{C} to PrP^{Sc} has not been definitively determined, so unknown variables in complex matrices may conceivably alter or prevent conversion from occurring.

The development of prion inactivation technologies to reduce and/or eliminate the potential of infectious prions entering environmental reservoirs is of the upmost importance. Prions have been documented to persist in soil for several years (Brown and Gajdusek, 1991; Georgsson et al., 2006), and have been detected in water from a CWD endemic area in the United States (Nichols et al., 2009). Currently in Canada, specified risk material (SRM), i.e., tissues associated with nervous and immune systems in food animals in the production industry, pose an enormous problem. To limit the zoonotic spread of prions, the Canadian Food Inspection Agency enforces the removal of all SRM materials from cattle slaughtered for human consumption (Canadian Food Inspection Agency, 2010). However, due to economics and geographical constraints, the SRM from cattle (and other animals) is rendered and then disposed of in landfills (Gilroyed et al., 2010b). During rendering, temperatures reach ~95 °C (Taylor and Woodgate, 2003), which is not sufficient to destroy prions. Therefore, alternative inactivation technologies for handling large amounts of SRM that could also reduce or eliminate hazards associated with prions entering the environment would be extremely beneficial. One such potential inactivation technology under investigation in this work is anaerobic digestion (AD) of SRM. Treatment of SRM using AD has the value-added benefit of producing renewable biogas energy (Gilroyed et al., 2010a,b). However, detection of infectious prions from this matrix using assays such as Western blotting and ELISA was challenging, and thus the efficacy of anaerobic digestion in prion inactivation remained unknown.

The focus of this study was on the adaptation of the PMCA assay to prion detection in anaerobic digestate.

2. Materials & methods

2.1. Hamsters

Three to six week old Golden Syrian hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA) were used in all brain tissue preparations. The Canadian Council of Animal Care (CCAC-Canada) guidelines were strictly adhered to for all hamster handling protocols.

2.2. Brain homogenates

2.2.1. Infectious material

The 263 K hamster-adapted scrapie prion strain was propagated in Golden Syrian hamsters (Charles River Laboratories International, Inc., Wilmington, MA, USA). Hamsters were euthanized with carbon dioxide at the clinical onset of disease (~95–100 days post-inoculation). Hamster infected brain tissue was confirmed scrapie positive by both ELISA and immunohistochemistry (IHC), and the infectious titre ($10^{9.94}$ ID_{50}/g of brain tissue) determined by hamster infectivity endpoint titration carried out at the Canadian Food Inspection Agency — Ottawa Laboratory Fallowfield (CFIA-OLF). Infectious brain homogenate (IBH) was prepared to a 10% (w/v) concentration in 1× PBS buffer (Bio-Rad Laboratories, Mississauga, ON) with manual homogenization (potter/glass tissue grinder, Fisher Scientific, Ottawa, ON) and stored at −80 °C until use.

2.2.2. Non-infectious (normal) material

Uninfected Golden Syrian hamsters were sacrificed by exposure to excess carbon dioxide, followed by perfusion of cold PBS (1× PBS, Bio-Rad Laboratories, Mississauga, ON) with a final concentration 4 mM of EDTA (Gibco Invitrogen Canada Inc., Burlington, ON) through the circulatory system of the expired hamster via a needle puncture to the left ventricle of the heart using a peristaltic pump. Harvested brains were collected and immediately flash frozen on dry ice and stored at −80 °C. Ten percent (w/v) normal brain homogenate (NBH) was prepared similarly to the IBH by manual homogenization, but instead using a conversion buffer according to Castilla et al. (2006) with the following modifications: 0.66× PBS prepared using BioBasic Inc. (Markham, ON) tablets, with one tablet/150 mL Milli-Q water (final concentration 91 mM sodium chloride, 1.8 mM potassium chloride, 6.7 mM phosphate buffer), EDTA (Gibco Invitorgen Canada Inc., Burlington, ON) to a final concentration of 4 mM (Castilla et al., 2004) and sodium heparin (BD Vacutainer, Franklin Lakes, NJ, USA) at a final concentration of 15 USP. The 15 USP sodium heparin concentration was obtained by adding 10 mL of 0.66× PBS (BioBasic Inc., Markham, ON) to a pre-coated 150 USP sodium heparin vacutainer (BD Vacutainer, Franklin Lakes, NJ, USA).

2.3. Anaerobic digestion effluent

Anaerobic digestate was collected from 2 L digesters (Minifors, Infors AG, Switzerland) that were initially inoculated with fresh cattle manure slurry and SRM (30 g/L total organic matter, 75:25 wt./wt. manure:SRM). Digesters were operated at 37 °C with a hydraulic retention time of 30 d for 1 year prior to digestate sampling. Aliquots of digestate (50 mL) were taken, frozen at −80 °C, and shipped to Alberta Agriculture Containment Level III facility (Edmonton, AB).

2.3.1. Detection of PrP^{Sc} in the presence of anaerobic digestate

The purpose of this approach was to assess whether undefined components present in anaerobic digestate would interact with PrP^{C} or otherwise interfere with conversion of PrP^{C} to PrP^{Sc}. To achieve this, anaerobic digestate or Milli-Q water control was diluted in 0.66× PBS to a final concentration of 10 and 2% then combined at a 1:1 ratio with 20% (w/v) NBH (i.e., 2× conversion buffer components (Castilla et al., 2006), 8 mM EDTA, 30 USP sodium heparin in 0.66× PBS), resulting in a final concentration of anaerobic digestate (or Milli-Q water) of 5 and 1% in 10% NBH. Ten percent IBH was 10-fold serially diluted into the NBH in combination with concentrations of digestate or Milli-Q water for PMCA. In this scenario, the PrP^{Sc} present in IBH was not exposed to anaerobic digestate prior to the assay and therefore remained capable of converting PrP^{C} to PrP^{Sc}. However, the PrP^{C} present in NBH was exposed to anaerobic digestate and possibly susceptible to chemical or physical interactions with digestate components.

2.3.2. Bioavailability of PrP^{Sc} in anaerobic digestate

The purpose of this approach was to determine whether undefined components present in the anaerobic digestate would interfere with bioavailability of PrP^{Sc}, thus preventing it from converting PrP^{c}. We are defining bioavailability as the ability of PrP^{Sc} to convert PrP^{C} to the PrP^{Sc} form. Infectious brain homogenate IBH (10% w/v) was diluted to

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