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Persistence of the bovine spongiform encephalopathy infectious agent in sewage

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ABSTRACT

Horizontal transmission of prion diseases through the environment represents a considerable concern. Prions are extremely resistant to inactivation and are thought to enter the environment after burial of animal mortalities or through biosolids from wastewater treatment plants. In addition, deposition of prions in the environment through biological fluids and/or faeces has been proved in the last years. Little is known about the behaviour of prion infectivity in the environment. In this study, the persistence of BSE infectious agent in sewage has been assessed by both PrP^{Res} immunoblotting and mouse bioassay in a long-term incubation study. Results indicated that no PrP^{Res} was detected after 150 day of incubation and consistent with this, a statistical regression model estimated 2-logs decay in 151 day. In contrast, no reduction in infectivity was observed during this period. Similarly, BSE infectivity remained unaltered after incubation in PBS for 265 day, whereas PrP^{Res} levels dropped progressively over the length of the study. These results indicate that in sewage and PBS, prion infectivity persists longer and with different dynamics than its commonly used marker PrP^{Res}. Thus, mathematical models computed on the basis of PrP^{Res} detection were unable to predict inactivation of prion infectivity. It is also reasonable to assume that conventional wastewater treatments with low retention times could have a very limited impact on prion infectivity. This data is essential for the development of accurate risk assessment analysis for BSE and other prion diseases in the environment.

1. Introduction

Transmissible spongiform diseases (TSEs) are a group of fatal neurodegenerative disorders that includes Creutzfeldt–Jakob disease (CJD), Gerstmann–Straussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI) in humans, bovine spongiform encephalopathy (BSE) in cows, scrapie in sheep and goats, and chronic wasting disease (CWD) in deer and elks (Prusiner, 1998). They are characterised by the conversion of a host protein, known as PrP^{c}, into its pathogenic form, PrP^{Sc}, which catalyses the conversion of new PrP^{c} molecules into PrP^{Sc}, leading into the accumulation of proteinase K-resistant PrP (PrP^{Res}) and constituting the causative agent of prion diseases (Aguzzi et al., 2008).

Prion diseases can have sporadic, heritable or infectious aetiology. Infectious prions originate from the ingestion of contaminated aliments (oral transmission) or from direct contact with contaminated surgical instruments (iatrogenic transmission). Scrapie and CWD prions are in addition thought to transmit horizontally through the environment (Miller and Williams, 2003; Williams, 2005). In the past years, shedding of prions in biological fluids and faeces during an animals' life time has been proved, thus supporting prion contamination of the environment. CWD prions have been detected in saliva (Haley et al., 2009; Mathiason et al., 2006), urine (Gonzalez-Romero et al., 2008; Haley et al., 2009), faeces (Safar et al., 2008; Tamguney et al., 2009), and even in the potential source tissues, such as salivary gland, urinary bladder and distal intestinal tract (Haley et al., 2011). Sheep scrapie prions have also been identified in saliva (Vascellari et al., 2007), urine (Rubenstein et al., 2011) and faeces (Terry et al., 2011). More importantly, sheep and CWD prions are known to resist in the environment for long periods of time. Scrapie infectivity persisted at least for 3 years after intentional

Abbreviations: BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; TSE, transmissible spongiform encephalopathies; PrPc, cellular prion protein; PrPSc, scrapie-associated prion protein; PrPRes, protease-resistant prion protein

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burial in soil (Brown and Gajdusek, 1991) and for more than 16 years in a naturally contaminated sheep house (Georgsson et al., 2006). CWD prions remained for at least 2 years in the soil of a farm that had housed infected animals (Miller et al., 2004). Interestingly, Johnson et al. have proven that prion infectivity in soil remains unaffected, or even enhanced, and can be accessible for other individuals to become infected through the oral route (Johnson et al., 2007,2006). Collectively, these data depict a scenario where persistence of prions in the environment may occur due to direct shedding from pre-clinical and clinical animals and remain infectious in soil for periods of time long enough to permit transmission to susceptible individuals. This prion accumulation in the environment has recently been demonstrated by the detection of PrP^{Sc} in water from an endemic CWD area (Nichols et al., 2009).

The scenario described for scrapie and CWD does not apply to other TSEs. BSE prions are hardly detectable in extraneural tissues and are essentially restricted to the CNS (Buschmann and Groschup, 2005; Espinosa et al., 2007). Thus, a significant shedding during pre-clinical stages seems unlikely in these cases. In agreement, they are not known to be horizontally transmitted through the environment (Anderson et al., 1996). Conversely, deposition of BSE prions in the environment may occur due to burial of carcasses and to a lesser extent, through biosolids generated in water treatment plants processing infected animals, especially those being unaware of it. Presumably this scenario occurred during the BSE epidemics (Saunders et al., 2008). BSE is also known to be the origin of the variant of CJD (vCJD), a fatal human prion disorder with a remarkably aggressive phenotype and unusual short incubation periods (Will et al., 1996), acquired by the consumption of BSE-contaminated beef products (Bruce et al., 1997; Hill et al., 1997). Thus, BSE prions in the environment could potentially transmit to animals as well as humans, although the calculated risk of such transmission remains extremely low.

Scrapie and CWD agents in the environment clearly represent a hazard. Prions can contaminate the environment through discharges of raw or partially treated wastewater and biosolids (Hinckley et al., 2008); they can reach urban wastewater facilities by leaching from landfilling or other disposal practices for TSE-contaminated waste (Hinckley et al., 2008; Pedersen et al., 2006); and they can potentially be reintroduced in the environment after land application of biosolids derived from wastewater facilities receiving TSE-contaminated waste (Gale and Stanfield, 2001; Gale et al., 1998). Understanding the fate of prions in wastewater treatment processes is important to minimise prion contamination and to control the spread of TSEs. In the past years, several studies have assessed the persistence of PrP^{Res} in different water environments (Hinckley et al., 2008; Kirchmayr et al., 2006; Maluquer de Motes et al., 2008) and more recently, cell culture has been applied to determine the survival of infectious mouse-adapted prions in water and biosolids (Miles et al., 2011a,2011b). However, to date there are no reports about the behaviour of BSE infectivity in wastewater. In this study, the persistence of BSE infectious agents in wastewater has been analysed comparatively by both PrP^{Res} detection and mouse bioassay. Data obtained will help the design of risk assessment studies aimed at determining the role of BSE and other TSE diseases as environmental contaminants.

2. Materials and methods

2.1. BSE-infected tissue

The BSE inoculum used in this study was prepared from a pool of brainstem from 49 BSE-infected cattle. Brains were homogenised in phosphate buffered saline (PBS) to give a 10% (w/v) inoculum, which contained around 10^7 infectious dose 50 (ID50) g^{-1} tissue (Castilla et al., 2003).

2.2. Sewage

Raw sewage was collected at the entry of a water treatment plant in Barcelona (Spain) that receives approximately 525,000 m^3 of urban sewage per day. Samples were collected in sterile 1-L polyethylene containers and kept at 4 °C before their use. Urban sewage samples had been routinely monitored in our laboratory to determine the level of faecal contamination by analysing the concentration of human adenoviruses (HAdVs) using quantitative PCR (Albinana-Gimenez et al., 2009; Bofill-Mas et al., 2006; Hundesa et al., 2006). The concentration of Enterococci in these sewage samples ranges between 10^5 and 10^7 colony-forming units (CFU) L^{-1} and the HAdVs titre by quantitative PCR is between 10^5 and 10^8 genome copies (GC) L^{-1}. Also, biochemical parameters, like pH and turbidity, are around pH 7.2 and 269 nephelometric turbidity units (NTU).

2.3. Incubation of BSE in sewage

Two sterile glass bottle containing either 250 mL of sewage or 250 mL of PBS were spiked with 5.0 ml of the described BSE inoculum and kept static under natural daylight regime at 20 °C (± 2) during the whole experiment. Aliquots of 12 mL from each of the 4 bottles were sequentially collected within a 265-days period on days 0 (30 min after), 7, 21, 45, 90, 120, 150, 180, and 265. Jars were thoroughly mixed before sampling and aliquots were immediately concentrated as described before (Maluquer de Motes et al., 2008). Briefly, samples were ultracentrifuged (100,000*g* 1 h 4 °C) and the pellets resuspended in 1 mL 0.25 N glycine (pH 9.5). Pellets were sonicated, incubated on ice for 30 min and centrifuged (2000*g* 15 min 4 °C). Supernatants were finally concentrated by ultracentrifugation to 100 μL PBS and stored at −80 °C until the experiment was completed. For identification purposes, samples were labelled BSE-T# or PBS-T# (where # corresponds to the days of incubation) according to the matrix employed, and are referred to as such throughout the study.

2.4. Proteinase K (PK) treatment and immunoblotting (IB) analysis

Twenty-five μL of each sample concentrate were treated with PK (Roche) for 1 h at 37 °C at a final concentration of 20 μg mL^{-1}. Laemmli buffer without reducing agents was then added and samples were boiled for 5 min before analysis by IB. Denatured samples were fractionated in sodium dodecyl sulphate polyacrilamide gels (SDS-PAGE, 12%) and transferred onto PVDF-membranes. Monoclonal antibody 2A11 (Brun et al., 2004) was used to immunoblot the membranes and these were finally visualised by the ECL-Plus developer kit (GE Healthcare).

2.5. Quantitation of PrP^{Res} and regression curves

In order to quantitate the amounts of PrP^{Res} detected using a non-quantitative method such as IB, films were transformed into digital pictures and the intensity of the immunoreactive bands was measured with Image J, an open-source image processing and analysis software distributed by the National Institutes of Health (http://rsb.info.nih.gov/ij/). The integrated density of the PrP^{Res} bands was plotted over time and regression curves were generated using the R package version 2.13.2 (R Development Core Team, 2011). Initially, we computed a linear regression model with the logarithm of the estimated concentration of PrP^{Res} expressed as integrated density. More precisely, the model is $\log y_t = \log y_0 + \alpha t$, where y_t is the mean of the values of y at time t, y_0 is the predicted value at time 0, and α is the slope of the regression line. The goodness of fit of the curves was assessed using R^2 values. To increase the confidence of the estimations, another model was computed using a quadratic regression curve. The equation of this second model is $y_t = y_0 + \alpha t + \beta t^2$ where y_0 is the predicted value at time 0, and α and β are the linear and quadratic term of the regression curve respectively.

2.6. Mouse bioassay

For mouse bioassay the Tg110 mouse line (Castilla et al., 2003) was used. BoPrP-Tg110 mice express bovine PrP at levels approximately 8-fold higher than cattle brain and are highly susceptible to BSE infection (Castilla et al., 2004,2003; Espinosa et al., 2007). Groups of 5–9 mice (6–7 weeks of age) were housed according to the guidelines of the Code for Methods and Welfare Considerations in Behavioural Research on Animals (Directive 86/609EC). Mice were inoculated in the right parietal lobe by using a disposable 25-gauge hypodermic needle. To diminish the risk for bacterial infection, the inoculated were preheated for 10 min at 70 °C before inoculation. 20 μL of each sample was delivered to each animal. Neurologic status of the inoculated mice was assessed twice a week. Animals were killed for ethical reasons when progression of the disease was evident or at the set end point (600 day post-inoculation). Once sacrificed, necropsy was performed