Amyloid-specific fluorophores for the rapid, sensitive in situ detection of prion contamination on surgical instruments

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Prion diseases or transmissible spongiform encephalopathies (TSEs) are a group of rare, transmissible and fatal neurodegenerative diseases associated with the protein agent (PrPSc). As such, the sensitive and rapid detection of prion PrPSc amyloid on the surface of suspect surgical instruments is of great importance and may even allow remedial action to be taken prior to any further operative intervention and possible iatrogenic transmission. However, conventional PrPSc detection methodologies tend to rely on the inefficient and unreliable removal of suspect material from a surface using swabs or wipes prior to antibody analysis. Here we show how the combination of an advanced light microscope technique, episcopic differential interference contrast/epifluorescence (EDIC/EF) microscopy, and the application of β-amyloid fluorescent thiazole markers (thioflavin T, thioflavin S) can be used to detect, in situ, submicron (attomole) levels of prion protein amyloid contamination in brain and spleen sections, smears and homogenate on surgical stainless steel surfaces and surgical instruments. This technique, although not specific to an amyloid type, can be used to verify that surgical instruments are substantially free from prion amyloid protein soiling and hence reduce the risk of iatrogenic transmission.

INTRODUCTION

In 1996 a new addition to the group of rare and fatal human transmissible spongiform encephalopathies was identified (Will et al., 1996). This addition was termed as variant Creutzfeldt–Jakob disease (vCJD), and has been linked with the consumption of bovine spongiform encephalopathy (BSE)-infected products in the late 1980s (SCENIHR, 2005). Although vCJD possesses similarities to sporadic CJD (sCJD), research has shown that certain organs outside the central nervous system can transmit the disease (ACDP/SEAC, 2003), a situation that has been considered to be minimal in spontaneous or idiopathic strains such as sCJD (Pauli, 2005). The incidence of vCJD deaths has decreased from a peak of 28 cases in 1998 to only 5 in 2005. The same number of cases also occurred in 2006 (UK CJD Surveillance Unit, 2006) and this plateau in incidences may give an indication that the disease will become endemic to the human population (Ironsside, 2006). However, with the recent evidence emerging that peripheral tissues and blood are capable of transmitting the disease then it is clear that iatrogenic Creutzfeldt–Jakob disease (iCJD) is potentially a major health problem, with the possibility of relatively large numbers of subclinical asymptomatic carriers of the disease being present in the UK population. A 2004 study reported the prevalence of prion protein in an anonymized study of 12 674 suitable appendectomy samples obtained from histopathology departments across the UK (Hilton et al., 2004). This report indicated that they had identified 3 samples that displayed an accumulation of prion protein and this produced an extrapolated prevalence of 237 per million (95% confidence interval 49–692 per million). Also, in 2004 it was reported that two patients, who had received blood products from asymptomatic vCJD blood donors, were found positive for the disease marker protein PrPSc. One of the recipients was MM homozygous at PRNP codon 129 and became new variant (nvCJD) symptomatic 6.5 years after transfusion (Llewelyn et al., 2004). The other subject was heterozygous at PRNP codon 129 and had died without symptoms of neurodegenerative disease (Peden et al., 2004). These discoveries lead the UK to ban blood donations from anyone who had received blood during the BSE crisis from 1980 and until 1996 (Ironsside, 2006).

Further cause for concern has been the recent publication (Peden et al., 2006) that aggregation of the prion protein occurs in the skeletal muscle of sufferers of different prion diseases. Peden et al. (2006) found accumulation in vCJD (8/17), sCJD (7/26) and iCJD (2/5) and concluded that these findings could have important implications for the potential risk of iatrogenic spread via contaminated surgical instruments.
Ever since the prion ‘protein only’ model was hypothesized (Prusiner, 1998), there has been scepticism concerning whether PrPSc is required for infection. Although recently there have been studies both for (Surewicz et al., 2006) and against (Hachiya et al., 2007) this hypothesis, a strong correlation between infectivity and PrPSc has been previously reported (Lee et al., 2001). Whichever theory is upheld, it has been shown that the prion infectious agent can remain infectious after exposure to traditional inactivation regimes such as autoclaving (121 °C, 30 min) or chemical solutions e.g. formaldehyde (ACDP/SEAC, 2003). The ability of prion infectivity to pass iatrogenically from subject to subject through surgical procedures has been proven both in the laboratory environment (Weissmann et al., 2002; Zobeley et al., 1999) and in healthcare institutions (Bernoulli et al., 1977; Collins & Masters, 1996). This problem is compounded by the lack of definition related to the disease incubation time and the fact that there is, at present, no detection system that is both sufficiently rapid to allow remedial intervention and sensitive to display small aggregates of the most reliable prion disease marker (PrPSc). These facets of the prion disease, in conjunction with the discovery of PrPSc in peripheral tissues (Bosque et al., 2005) and evidence of infectivity in blood (Hunter et al., 2002), have led to the increased fear of iatrogenic transmission and the greater need for a rapid, sensitive detection system to screen suspect items and assess the risk of cross contamination.

The detection of PrPSc in tissue is dependent on either Western blotting (Kascak et al., 1987), ELISA (Pan et al., 2005) or immunocytochemistry (Bell et al., 1997), none of which can be applied directly to the surface of an instrument. These techniques are time consuming, relatively insensitive and require the elution of any suspect material from a surface, with the artefacts that this might cause. In addition the majority of prion antibodies are not able to discriminate between the pathogenic isoform of the protein (PrPSc) and the normal cellular isoform (PrPc), requiring the introduction of a proteinase K (PK) step to preferentially degrade the more susceptible PrPc (Kovacs et al., 2002). Nevertheless, PrPSc still suffers degradation by PK, further reducing the sensitivity of the assay. Any changes to PrPSc epitopes may also reduce sensitivity of detection, depending on the specificity of the antibody used. Although some commercial screening tests such as Idexx HerdChek (Idexx Laboratories) and InPro CDI (InPro Biotechnology) do use little or no PK, such tests still require the sample to be in solution and, in the case of surgical instruments, would require the elution of any material from the device’s surface prior to testing. Therefore the ability to detect the PrPSc disease-linked marker in unstructured prion-positive tissue, in situ, on surgical instrument surfaces could both reduce the delay in instrument contamination assessment and be a useful tool in preventing iatrogenic prion transmission. The present study describes the development of a novel microscopy procedure, episcopic differential interference contrast/epifluorescence (EDIC/EF) microscopy in conjunction with sensitive thiazole fluorescent probes, thioflavin T and thioflavin S, to detect PrPSc directly on surgical stainless steel instruments.

For our studies of experimental detection of the prion agent we have used an ME7 (murine scrapie) model of prion disease. The ME7 agent has been known for some 25 years and the pathology and disease it produces have been well studied at the pathological and behavioural level (Betmouni & Perry, 1999; Bruce et al., 1976; Dell’Omo et al., 2002; McBride et al., 1988). The thiazole derivatives, thioflavin T and thioflavin S, have been advocated for the detection of amyloid and β-pleated sheet proteins (Guntern et al., 1992; Saeed & Fine, 1967) in both histopathology (Burns et al., 1967) and biochemical assays (Klunk et al., 2001). These derivatives have been reported to display an affinity to bind with aggregates of prion protein (Ishikawa et al., 2004) and in both sections and frozen homogenate slices (Klunk et al., 2001). However, the direct application and visualization of such dyes on surgical instruments and other stainless steel surfaces and the assessment of ‘realistic’, unstructured soiling, such as homogenates and smears has, to the best of our knowledge, never been reported. In this study we show how a relatively simple procedure can rapidly distinguish prion-infected surfaces from non β-amyloid contaminated substrata without the requirement to elute or remove the infectious material and the possible artefacts this removal may create.

### METHODS

**Microscopy.** The construction of the prototype microscope for episcopic differential interference contrast (EDIC) and epifluorescence (EF) studies involving non-contact with samples has been described previously (Keevil, 2003). The design alleviated the need for contact objectives and coverslips or oil immersion, facilitating rapid scanning of even curved and/or serrated surfaces such as surgical instruments (Lipscomb et al., 2006b).

**Stainless steel tokens.** The tokens (25 × 75 mm) were made from 316L surgical grade stainless steel and had passed through a modification of the cleaning process described previously (Lipscomb et al., 2006b). Briefly, the tokens were washed in acetone (Sigma-Aldrich) for 30 min at 121 °C before being vortexed in five changes of filtered distilled water. The tokens were loosely wrapped in aluminium foil and placed into an oven at 230 °C for 16 h. The tokens were examined using EDIC/EF microscopy prior to use to confirm cleanliness. This process is able to produce a ‘pristine’ surface free from any residual soiling prior to the artificial contamination studies.

**Surgical instruments.** Spencer-Wells haemostatic forceps were obtained from an anonymous NHS Trust sterile service department. To ensure that the forceps were free from proteinaceous contamination, the instruments were cleaned and assessed for deposits using a method described previously (Lipscomb et al., 2006b). No protein contamination was present before the instruments were deliberately contaminated with normal or prion-infected brain homogenate.

**Tissue sections.** Prion-infected brain material was obtained from female C57BL/6 J mice which had been injected with 1 μl 10% (w/v) ME7-infected brain homogenate, into the dorsal hippocampal region.
of the brain as described elsewhere (Betmouni & Perry, 1999). Control mice were injected with a normal brain homogenate (NBH) in a manner comparable to ME7-injected animals. All animals were then sacrificed at 19–21 weeks post-inoculation and none of the control group displayed signs of infection. The brain or spleen tissue was frozen in liquid N₂ and embedded in Lamb OCT embedding medium (Raymond Lamb Ltd) and thin (10 μm) coronal sections were cut using a cryostat (Bright Instrument Company) and placed onto the stainless steel tokens.

**Tissue smears and homogenate.** Tissue smears were produced by placing a freshly cut frozen section onto a cleaned metal token and applying a blood smear technique (Bancroft & Stevens, 1990). The steel tokens and surgical instruments were artificially contaminated by the application of 1 μl samples of 1 mg ml⁻¹ normal brain homogenate (negative controls) or ME7 brain homogenate (positive controls) and allowed to dry for 30 min at 37 °C, before staining.

**SDS-PAGE and Western blotting** The electrophoresis and blotting buffers were obtained from Bio-Rad. Brain homogenates from ME7 mice and control homogenate (TSE Resource Centre, Institute for Animal Health, Newbury, UK) were mixed in various proportions from 0 up to 100 % ME7, all normalized at 1 mg ml⁻¹, and subjected to digestion by PK (Sigma-Aldrich) to a final concentration of 10 μg (mg total protein)⁻¹, at 37 °C for 1 h. A serial dilution of recombinant PrP (Dr Andrew Gill, Institute for Animal Health, Newbury, UK) was used for calibration. Samples were boiled for 5 min in loading buffer containing 2 % SDS, 10 % glycerol, 2 % β-mercaptoethanol and 0.01 % bromophenol blue in 50 mM Tris/HCl, pH 6.8, and loaded on a 12 % polyacrylamide gel (Bio-Rad; brain homogenate equivalent to 1 μg per well, recombinant PrP ranging from 0.1 to 10 ng per well). After separation, proteins were transferred onto a PVDF membrane (Bio-Rad) which was blocked in 5 % non-fat dry milk in PBS (Invitrogen) containing 0.1 % Tween 20 (PBST; Sigma-Aldrich) for 1 h at room temperature (as all following steps). PrP was detected using the monoclonal antibody SAF 60 (Commissariat a’l’Energie Atomique, Saclay, France) diluted 1:5000 in PBST incubated for 1 h, followed by horseradish peroxidase-conjugated anti-mouse IgG (1:5000 in PBST; GE Healthcare) for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences).

**Thioflavin T staining.** Prion and normal brain tissue on steel tokens and surgical instruments were fixed in 100 % ethanol at 4 °C (w/v) for 10 min. After washing with PBS, the sections were incubated with thioflavin T [0.1 % (w/v) solution in 0.1 M hydrochloric acid] for 10 min at room temperature. The tokens were subsequently washed with 1 % (v/v) acetic acid in deionized water for 10 min, then with deionized water for 30 min.

**Thioflavin S staining.** Sections of fresh tissue on steel tokens were fixed in 4 % (w/v) paraformaldehyde at 4 °C for 10 min. After washing with PBS, the sections were incubated with thioflavin S (Sigma-Aldrich) (0.01 % (w/v) solution in deionized water) for 10 min at room temperature. The samples were then washed in 10 min steps with decreasing alcohol concentrations, 100, 90, and 70 % (v/v), and finally rinsed for 10 min in deionized water.

**RESULTS**

**Detection of PrPSc in brain tissue on surgical steel tokens**

Initial work investigated whether the thiazole derivatives, thioflavin T and thioflavin S, could be used to detect PrPSc on stainless steel tokens. Brain tissue sections (10 μm) were placed onto steel tokens and stained with the thiazoles. The large plaques of PrPSc and ‘mini-plaques’ with a diameter of less than 1 μm were readily visible using EDIC/EF microscopy (Fig. 1) in a virtually identical pattern to adjacent sections of brain material stained by immunocytochemistry using the 6H4 mAb (Prionics AG) in conjunction with standard formic acid and hydrated autoclaving pre-treatments (Brown et al., 2000; Liu et al., 2003) (data not shown).

**Fig. 1.** Photomicrographs showing PrPSc-positive regions (white) in brain sections compared with unstained areas in (a) NBH section (bar, 100 μm), (b) ME7-prion section stained with thioflavin S (bar, 100 μm), (c) ME7-prion section stained with thioflavin T (bar, 100 μm) and (d) ME7-prion section stained with thioflavin T (bar, 30 μm). All sections were mounted on stainless steel tokens.
It was observed that thioflavin T (Fig. 1b) displayed a superior signal to noise ratio compared with thioflavin S (Fig. 1b) when bound to amyloid, and exhibited a useful Stokes shift in emission wavelength from 450 nm to 480 nm, whereas thioflavin S did not. It was therefore used in all the subsequent experiments.

**Detection of PrP<sup>Sc</sup> in spleen and non-uniform brain tissue contamination on surgical steel tokens**

Similar results were obtained for PrP<sup>Sc</sup>-infected spleen sections on stainless steel and confirmed the sensitivity of PrP<sup>Sc</sup> amyloid detection for this tissue type (Fig. 2). In addition, a positive fluorescence signal could clearly be differentiated in both tissue smears and homogenates of PrP<sup>Sc</sup>-infected brain material on the stainless steel coupons (Fig. 3). There was no thiazole staining of comparable NBH material (data not shown).

**Detection of PrP<sup>Sc</sup> in brain tissue on surgical instruments**

A similar positive fluorescence signal was seen on the surgical instruments contaminated with PrP<sup>Sc</sup>-infected brain tissue (Fig. 4a), but not with NBH (Fig. 4b). The surface of the surgical instruments can be seen to be quite rough when viewed with EDIC microscopy in the white light channel and it proved impossible to visualize low level contamination without the thiazole fluorophore and observation with the EF channel of the microscope.

**Comparison of sensitivity to conventional Western blot techniques**

SDS-PAGE electrophoresis and Western blotting were performed in parallel with staining and EDIC/EF microscopy to compare the sensitivity of the two methods for the detection of PrP<sup>Sc</sup>. The initial protein concentrations of the 10% brain homogenates were 15.8 mg ml<sup>-1</sup> (ME7) and 21.6 mg ml<sup>-1</sup> (NBH). These were normalized to 1 mg ml<sup>-1</sup> in deionized water, and the same amount that was applied on tokens was loaded in the gel for comparison (1 µl), i.e. each lane contained 1 µg of brain homogenate. The lower limit of detection of pure recombinant PrP in our Western blot assays was 1 ng (Fig. 5).

Following partial digestion with PK, the samples containing only ME7 produced clear bands characteristic of PrP<sup>Sc</sup> (Fig. 5). The mixes containing 10% ME7 (equivalent to 0.1 µg of protein from ME7 brain) rarely produced visible bands, and the 1% ME7 mixes (equivalent to 10 ng of protein from ME7 brain) were never distinguishable from the NBH control. In comparison at the same concentration a positive signal was still readily observable from the EDIC/EF microscope analysis (Fig. 6) and the findings show this technique both to allow direct visualization of the contamination and to be at least twofold more sensitive than Western blotting techniques.

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**Fig. 2.** Photomicrographs showing PrP<sup>Sc</sup>-positive regions (white) in spleen sections compared with unstained areas in (a) NBH section, (b) ME7-prion section stained with thioflavin T. Bars, 100 µm.

**Fig. 3.** Photomicrograph of thioflavin T-stained PrP<sup>Sc</sup> tissue contamination on stainless steel tokens, (a) smear, (b) homogenate. Bars, 10 µm.
Theoretical sensitivity of detection limit

By applying the known biological characteristics and physical dimensions of the mouse brain (Table 1), we were able to provide an estimate of the amount of PrPSc protein that might be contained within one of these 1 μm diameter mini-plaques, which are readily visualized within the brain tissue. By modelling the cross-sectional area of a section as oval and assuming deposits to be spherical in nature, then the known murine brain characteristics and

Table 1. Biological and physical attributes of a murine brain applied to calculate the in situ sensitivity of thioflavin T-stained PrPSc protein

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain weight (whole brain)</td>
<td>482.3 mg (Average obtained from 129 mice within 23 litters)</td>
<td>Mouse Brain Library (2002)</td>
</tr>
<tr>
<td>Percentage of protein in brain</td>
<td>12 % (Davis &amp; Himwich (1973); Folch Pi (1955))</td>
<td></td>
</tr>
<tr>
<td>Diameter of deposit</td>
<td>1 μm</td>
<td></td>
</tr>
<tr>
<td>Average overall brain dimensions (n=47)</td>
<td></td>
<td>Franklin &amp; Paxinos (1997)</td>
</tr>
<tr>
<td>Anterior–posterior (Xav)</td>
<td>11 mm</td>
<td></td>
</tr>
<tr>
<td>Dorsal–ventral (Dav)</td>
<td>5.5 mm</td>
<td></td>
</tr>
<tr>
<td>Medial–lateral (Lav)</td>
<td>8 mm</td>
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dimensions (Table 1) can be applied using the equation given below (equation 1) to calculate the protein in a 1 μm diameter aggregate. These calculations indicate that the thioflavin T-positive, 1 μm aggregates which are clearly visible, are composed of less than 100 fg protein, which equates to around 2 attomoles PrPSc.

\[ P_d = \frac{V_s}{(4/3) \pi r^3} \]

where:
- \( P_d \) is the amount of protein in the whole brain
- \( V_s \) is the volume of deposit (mm³)
- \( r \) is the radius of the aggregate

The initial calculation of protein concentration of brain material was confirmed using a commercial total protein assay (Bio-Rad).

**DISCUSSION**

In the UK the recommended assessment method for surgical instrument decontamination is the application of the ninhydrin reaction (NHS Estates, 1997). This reaction relies on the removal of material from an instrument’s surface using a swab, and a subsequent colorimetric chemical reaction. However, such sampling techniques have been shown to be subject to inconsistency (Lipscomb et al., 2006a; Spongiform Encephalopathy Advisory Committee, 2005).

To overcome these drawbacks in sensitivity, previous studies have developed a sensitive and rapid general SYPRO Ruby method (Lipscomb et al., 2006b), which has been shown to be over 1000-fold more sensitive than ninhydrin (Lipscomb et al., 2006a) to assess post sterile service department-processed surgical instruments (Lipscomb et al., 2006c, d). The results have shown that large numbers of surgical instruments are still extensively contaminated with protein deposits (on average between 0.42 and 4.2 μg) even after passing through the recommended cleaning and decontamination procedures. If only part of these proteinaceous deposits were prion, then they would pose a serious clinical risk of cross contamination and iatrogenic transmission. However, the SYPRO Ruby test cannot discriminate whether prion amyloid is actually present and an important step forward, reported here, has been the development of this new rapid test for amyloid detection.

Sensitive Western blotting assays using sodium phosphotungstic acid precipitation have been reported to allow the detection of PrPSc from 5 nl of 10% vCJD brain homogenate (Wadsworth et al., 2001). This equates to 500 ng of tissue prior to PK digestion. We standardized our samples according to the protein content of the brain tissue, so comparison between the different methods can only be made based on volumes. Following initial dilution of our 10% ME7 homogenate down to our standard working protein concentration of 1 μg ml⁻¹ (1 in 15.8 dilution), we applied 1 μl homogenate in each lane. Using the monoclonal antibody SAF 60, we were able to detect PrPSc after partial PK digestion of 1 μg (equivalent to 63.3 nl) 10% ME7 homogenate. Only rarely a weak positive signal was detected after partial PK digestion of 100 ng or less (i.e. 6.33 nl) of ME7 homogenate mixed with NBH to make up the 1 μg total protein load. Considering we used a different species and a different prion strain, in combination with a different antibody, we were satisfied with the sensitivity of our standard Western blotting assay. In addition, the sensitivity of our detection of pure recombinant PrP (1 ng) was consistent with, if not better than, that of other groups using the same method (Appel et al., 2001).

The novel test method applied here is at least two log more sensitive than conventional Western blotting procedures and can visualize submicron and subpicogram levels of amyloid contamination without requiring the elution of suspect material. It also overcomes the recognized problem of susceptibility of even PrPres to PK degradation and epitope destruction, which reduces the amount of PrPres being loaded onto the gel and the sensitivity of the antibodies used for detection. This new in situ thiazole staining procedure will allow future surveys of surgical instruments to screen quickly and easily not only for general protein contamination, but also for more specific amyloid contamination. This will give greater insight into the extent of amyloid contamination across a range of surgical instruments and also permit an evaluation of improved cleaning and decontamination procedures that could be brought into general use.

Prion-specific detection methods tend to rely on Western blotting (Kascak et al., 1987), ELISA (Pan et al., 2005) or immunocytochemistry (Bell et al., 1997) and the application of PrP-targeted antibodies, although the ‘gold standard’ method of assessing tissue infectivity is the application of relatively lengthy animal studies (Cooley et al., 2001). None of these techniques can be applied directly onto surgical instruments, leading to the requirement that any suspect tissue must first be removed from the surface prior to any assessment. Any such sampling procedure would both increase the risk of artefacts in the results and reduce the overall sensitivity of the testing method (Lipscomb et al., 2006b).

In previous studies, thioflavin T derivatives have been shown to bind to prion protein aggregates (Ishikawa et al., 2004) and have been reported to possess both a high affinity for amyloid and the ability to cross the blood brain barrier (Klunk et al., 2001). These attributes are useful for drug therapy, but for the assessment of instrument contamination a large signal response and a detectable Stokes’ shift are of higher priority. Unfortunately, thioflavin S and derivatives of thioflavin T appear to show no spectral shift.
(λex/λem 340/440 nm) after binding, unlike thioflavin T itself (Lockhart et al., 2005) (λex/λem 353/444 nm unbound, 450/480 nm bound). In addition, the increase in fluorescence signal from thioflavin T after binding is considerably higher than that of thioflavin S (12.5-fold increase compared with 0.78-fold increase, respectively) (Klunk et al., 2001). These factors mean that thioflavin T is a more suitable candidate for PrPSc protein contamination detection on surgical instruments than other described derivatives.

In this paper we have demonstrated that the use of EDIC/EF microscopy and fluorescent dyes can detect submicron and subpicogram (attomole) levels of PrPSc contamination, in situ on surgical stainless steel, without the requirement for coverslips or oil immersion. This research has also demonstrated that the application of this in situ visualization method is considerably, at least twofold, more sensitive than conventional Western blotting techniques and can theoretically detect attomole levels of the infectious disease marker PrPSc.

The thiazole dyes do not discriminate between amyloid plaques composed of PrPSc and plaques that might be composed of other amyloidogenic fibrils such as Aβ, a major component of Alzheimer’s disease pathology. The possibility that Aβ fibrils may also display some infectivity (Meyer-Luehmann et al., 2006; Riek, 2006), even if such transmission is highly unlikely and of a different nature to prion disease, must raise concern over any surface-bound amyloid contamination that may be found on an instrument. The simplicity of this in situ staining and the rapid nature of its application lend it to the further development of this methodology in a wide variety of settings, and may prove valuable in the detection or confirmation of the presence of amyloid material such as PrPSc before or after the instrument has passed through traditional cleaning processes. The work here demonstrates the ability to detect small quantities of amyloidogenic plaques on surgical stainless steel surfaces after soiling by brain smears and homogenate, and opens the way to improved screening and decontamination protocols to prevent iatrogenic transmission of neurodegenerative agents such as PrPSc.

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