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A rapid dual staining procedure for the quantitative discrimination of prion amyloid from tissues reveals how interactions between amyloid and lipids in tissue homogenates may hinder the detection of prions

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ABSTRACT

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases with no cure to this day, and are often associated with the accumulation of amyloid plaques in the brain and other tissues in affected individuals. The emergence of new variant Creutzfeldt–Jakob disease, an acquired TSE with a relatively long asymptomatic incubation period and unknown prevalence or incidence, which could potentially be iatrogenically transmitted, has prompted the need for sensitive and rapid methods of detection of the pathology indicator, the protease-resistant prion protein (PrP^{Sc}), in tissues and on surgical instruments. To discriminate between common tissue proteins and amyloid-rich aggregates such as those formed by abnormal prion, we developed a quantitative thioflavin T/SYPRO Ruby dual staining procedure, used in combination with episcopic differential interference contrast/epifluorescence (EDIC/EF) microscopy for rapid scanning of samples. The detection limit of this direct observation technique applied to brain homogenates was greatly enhanced by the addition of Tween 20, as demonstrated in double-blind studies using various proportions of ME7-infected brain mixed with normal brain homogenate. The characteristic thioflavin T signal correlated with the relative amount of prion amyloid and proved at least 2-log more sensitive than the classic Western blot using the same prepared samples. This new sensitive microscopy procedure, which can be easily applied in instrument decontamination surveys, is likely to be more sensitive that Western blot in practice since it does not rely on the elution of resilient PrP^{Sc} bound to the instrument surfaces. Our study also demonstrates how interactions between prion and lipid-rich tissue homogenates may reduce the sensitivity of such detection assays.

1. Introduction

Transmissible spongiform encephalopathies (TSEs) are rare, fatal neurodegenerative diseases which may occur in various animal species (see reviews by Brown, 2008; Collinge, 2001; Prusiner, 1998). TSEs may develop when the host-encoded constitutive prion protein (PrP^{C}) is mutated to an isoform richer in beta-pleated sheets, which is partly resistant to proteolytic digestion. In humans, identified TSEs include Creutzfeldt–Jakob disease (CJD), Kuru, Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI), and these have been further classified according to their etiology (Gambetti et al., 2003). The incidence of a genetic mutation leading to sporadic TSEs is believed to be low (about 1 per million annually for sporadic CJD). According to the "prion only" hypothesis, absorption of exogenous PrP^{Sc} is sufficient to trigger the post-translational refolding of endogenous PrP^{C} in healthy, genetically susceptible individuals, leading to an acquired form of the disease. The accumulation of protease-resistant PrP^{Sc} in the form of amyloid plaques in tissues, particularly in the lymphoreticular and central nervous systems, are a common pathological feature of TSEs.

The consumption of contaminated tissues has been identified as a route of transmission leading to acquired CJD. This horizontal transmission of the disease was described in the cannibalistic Fore tribe of Papua New Guinea in the middle of last century (see reviews by Ironside, 2003; Collinge et al., 2006), and there is strong evidence that the same route was implicated in the bovine spongiform encephalopathy (BSE) epidemic originating from the UK during the mid 1980s, in which case cattle were fed on reprocessed carcasses of slaughtered contaminated animals ("meat and bone meal"), leading to the contamination of an estimated 1 million animals destined to human consumption (Wells et al., 1991; Anderson et al., 1996). There has been increasing experimental evidence that various protease-resistant prion strains are capable of crossing the species barrier (Mishra et al., 2004; Priola and Vorberg, 2004; Foster et al., 1993), and that the ingestion of BSE-infected beef meat is the actual cause of the so-called variant form of CJD in humans (vCJD; Hill et al., 1997). Acquired TSEs are characterized by a relatively long and initially asymptomatic incubation period (up to 50 years for Kuru; Collinge et al., 2006) which

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varies depending on the nature of the infective strain, the route of transmission, and the genetic susceptibility of the recipient. This has led to the fear that an unknown proportion of the population who have been eating potentially contaminated meat in the 1980s might currently present as asymptomatic carriers of the vCJD infectious agent, despite current statistics which indicate that the number of deaths by vCJD peaked in 2000 and has been in decline since (Caramelli et al., 2006; Setbon et al., 2005).

Beside transmission of the infectious agent through the ingestion of contaminated products, bearing in mind the potential threat of vCJD incubating in the population, a number of medical procedures are now also considered as posing a potential risk of iatrogenic contamination with PrP^{Sc} (Pana and Jung, 2005; Lumley and Serious Hazards of Transfusion Committee, 2008). The procedures identified at risk include neurosurgery and eye surgery (Dinakaran and Kayarkar, 2002), and other invasive interventions such as blood transfusion (Ironside, 2003; Llewelyn et al., 2004; Wroe et al., 2006), endoscopy (Ponchon, 1997; Axon et al., 2001) and dentistry (Azarpazhooh and Leake, 2006; Whitworth, 2007). In the absence of a cure for the disease, avoidance and precautionary measures are the only ethical option currently available to public and private health services. PrP^{Sc} is known to be resistant to most inactivation and decontamination protocols, and current methods of neutralisation in the laboratory involve strong alkali compounds (Jung et al., 2005), which are themselves detrimental to most surfaces. In recent years great emphasis has been put on the decontamination of surgical instruments as a means of preventing iatrogenic transmission, and a number of cleaning products have been developed and marketed for the purpose of tackling the protease-resistant prion issue, though relatively little has been done to assess the efficiency of these cleaners in a clinical context, where the turnover of surgical instruments is high and the time to clean them is limited. We have already reported that common methods currently available to assess the cleanliness of surgical instruments, such as direct visual inspection and indirect Biuret or ninhydrin chromogenic assays, are far from being sensitive at picking up remaining contamination on surgical stainless steel surfaces (Lipscomb et al., 2006a, 2008). We have previously described a new microscopic technique, episcopic differential interference contrast/epifluorescence (EDIC/EF) for the rapid and sensitive *in situ* detection of general protein contamination on surgical instruments (Lipscomb et al., 2006b), and more recently we have modified this technique for the detection of amyloid-like proteins using fluorescent thiazole reagents (Lipscomb et al., 2007). At present no other molecule or microorganism than PrP^{Sc} have been clearly associated with TSEs, despite recent reports on infective tissues that appear PrP^{Sc}-free. The presence of amyloid aggregates as those which may be seen with PrP^{Sc} are therefore a useful, if not the only, preclinical indicator of a potential pathology. Since PrP^{Sc} appears to bind strongly to surfaces, the detection of amyloid aggregates among other proteinaceous contamination on surgical instruments might serve as a useful tool in the prevention of iatrogenic dissemination of TSEs. In addition, the detection of PrP^{Sc} or other amyloid aggregates in tissues remains the best approach for clinical diagnosis of TSEs and other amyloidoses, though this is most often performed *post-mortem* via Western blot or immunohistochemistry.

Although the presence of amyloid aggregates is admittedly only a pathological indicator and does not directly correlate with the potential infectivity of a particular sample, a sensitive method to ensure that reused surgical instruments are free of general protein soil, especially detectable amyloid-containing deposits, would be a major advance in the prevention of potential iatrogenic contamination during surgical procedures. Accordingly, we have developed a new dual staining procedure which allows the rapid and sensitive quantification of amyloid content in proteinaceous deposits. Here we show the potential of this technique applied to the detection of the pathological amyloid aggregates in sample tissues, and show how tissue lipids may be a factor contributing to reduced sensitivity of such direct detection assays. We also demonstrate how this method may be used to evaluate decontamination practices using test surgical instruments spiked with better defined test soils, in terms of protein and amyloid concentration.

2. Methods

Preparation of samples for the determination of the lowest limit of detection of amyloidogenic prion in homogenates.

Brain homogenates from ME7-infected mice and control normal brain homogenate (NBH; both from TSE Resource Centre, Institute for Animal Health, Newbury, UK) were assayed and normalized to a protein concentration of 1 mg ml^{-1} in deionised water with 0.1% (v/v) Tween 20 (Sigma) to improve homogenisation for calibration (see results), aliquoted and stored at −20 °C until use. On the day of each experiment, ME7-infected homogenate and NBH stock aliquots were mixed in various proportions (from 0 up to 100% ME7 in NBH), anonymised, and kept in ice until staining and observation (usually within 1 h). One sample containing NBH only was clearly labelled as "negative control". Surgical stainless steel tokens were thoroughly cleaned as described previously (Lipscomb et al., 2007), and a 1 µl drop of each anonymised sample and the negative control was applied and dried on the tokens surface for 30 min at 37 °C prior to staining.

2.1. Dual staining procedure

We developed a dual staining protocol based on our previously described methods for the rapid detection of general protein contamination (Lipscomb et al., 2006b) and the specific detection of amyloid-like formations on surgical instruments (Lipscomb et al., 2007), with slight modifications. All incubations were performed at room temperature, and samples were protected from direct light as much as possible to prevent fading of the fluorescent signal. A thioflavin T (ThT; Sigma) working solution (0.2% w/v) was prepared shortly before use in 0.01 M hydrochloric acid, and applied onto samples for 10 min. Samples were washed by gentle immersion (no flushing) and incubated for 10 min with 0.1% (v/v) acetic acid, followed by a wash in PBS and another wash in deionised water. SYPRO Ruby solution (SR; Invitrogen, UK) was then applied for 15 min, followed by 3 final washes in deionised water. Excess water was dried off and samples were microscopically examined immediately after staining under an EDIC/EF microscope.

2.2. Microscopy

EDIC/EF microscopy and its application for the detection of proteinaceous deposits on surfaces have been described previously (Keevil, 2003; Lipscomb et al., 2006b, 2008). Volunteers were asked to examine each anonymous sample in two steps. Firstly, a scan of the whole area of surgical stainless steel covered by each sample was performed at 100× magnification using the SR filter set (excitation: 470 nm; emission: 618 nm; Nikon) to assess the total amount of protein still present after the dual staining procedure. Secondly, observers were asked to repeat the observation under 1000× magnification and select 10 areas for sampling and analysis, excluding relatively big tissue aggregates if present. Images of these areas were then captured using a CCD camera for both the ThT (excitation: 450 nm; emission: 480 nm; Nikon) and SYPRO Ruby signals. For each sample 10 areas of interest were used for further analysis.

2.3. Image analysis

Images were analysed using the ImagePro plus software (Media Cybernetics, Bethesda, MD, USA). To ensure that the sampling was homogeneous and the same amount of tissue was observed in each sample, the total amount of protein observed was estimated from the SR staining. For each experiment, the minimum threshold was

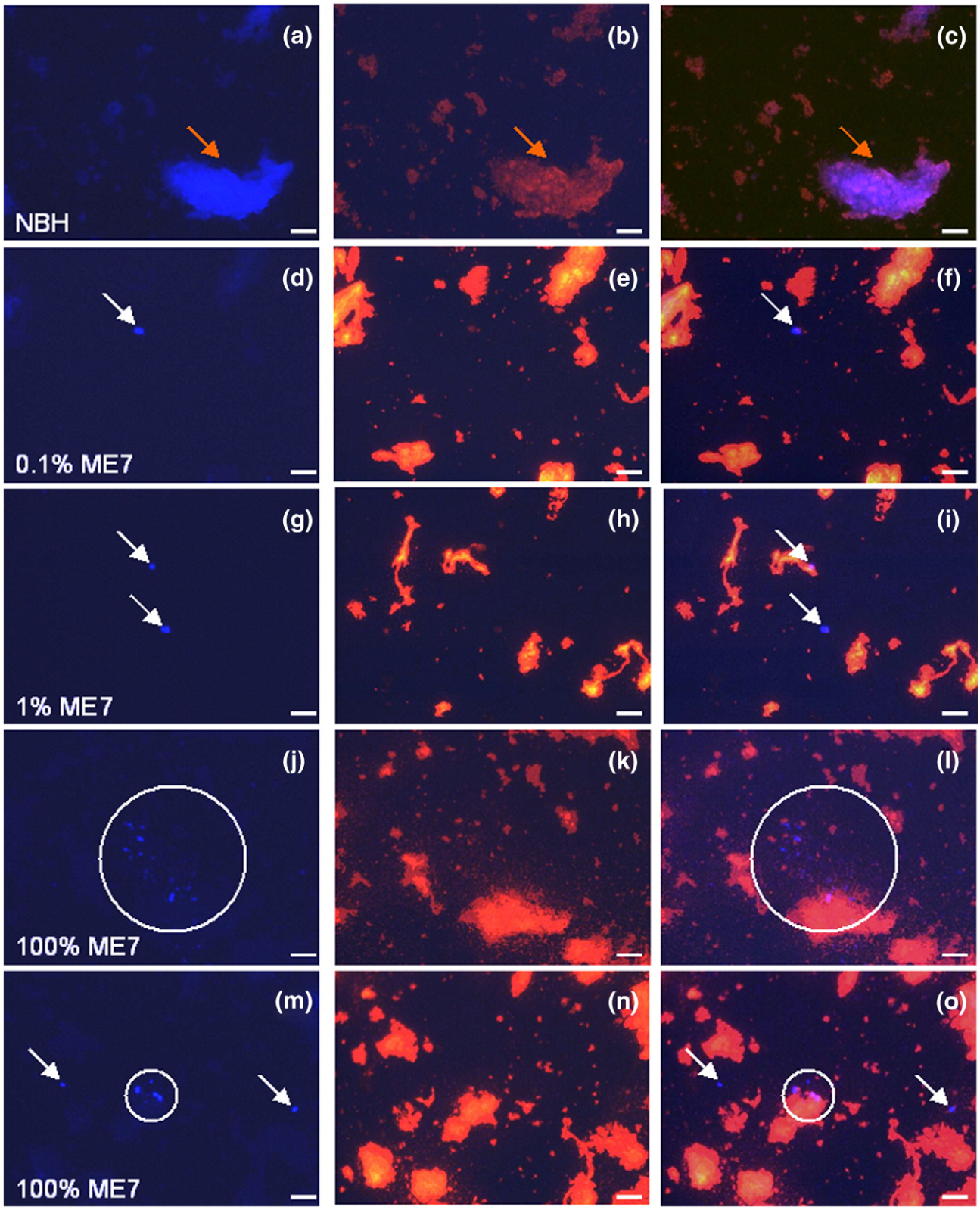

Fig. 1. Epifluorescence micrographs from thioflavin T staining (left column), SYPRO Ruby staining (middle column) and merged pictures (right column) of both; (a–c) NBH before addition of Tween 20 showing non-specific binding of ThT to large protein aggregates (red arrows). (d–o) effect of adding Tween 20 to abolish non-specific binding of ThT to non-amyloid proteins while showing discrete binding of ThT to small amyloid aggregates present in ME7-containing mixes. This produced the characteristic shift in thioflavin T emission to 482 nm and was clearly distinguishable once corrected for background. Positive areas as assessed by image analysis are indicated by white arrows and circles. Bars: 10 µm.

determined according to the intensity of the ThT signal in the known negative control. Unknown samples were subsequently analysed using that set threshold, hence only the stronger characteristic fluorescence of ThT bound to focal amyloid accumulation was considered as positive signal by the software. Data in pixels per area of surface were converted to pg mm^{-2} according to the estimation of

Lipscomb et al. (2006b) that a 1 µm-diameter area of protein corresponds to approximately 1 pg.

2.4. SDS–PAGE and Western blotting

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^{Sc} as described before (Lipscomb et al., 2007). Briefly, the same amount of ME7 and NBH homogenate mixes (all containing 1 mg ml^{-1} of protein) that was applied on tokens was loaded in the gel for comparison (1 µl), i.e. each lane contained 1 µg of protein from brain homogenate. The electrophoresis and blotting buffers were obtained from Bio-Rad, UK. Anonymised samples were subjected to digestion by proteinase K (PK, Sigma) to a final concentration of 10 µg PK per mg of total protein, at 37 °C for 30 min. Samples were boiled for 5 min in loading buffer (2% SDS, 10% glycerol, 2% β-mercaptoethanol, 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 of protein per well). After separation, proteins were transferred onto a PVDF membrane (Bio-Rad) which was blocked in 5% (w/v) non-fat dry milk in PBS (Invitrogen) containing 0.1% (v/v) Tween 20 (Sigma) (PBST) for 1 h at room temperature (as all following steps). PrP was detected using the monoclonal antibody SAF 60 (CEA, Saclay, France) diluted 1/5000 in PBST and incubated for 1 h, followed by HRP-conjugated anti-mouse IgG (1/10,000 in PBST; GE Healthcare, UK) for 1 h. Immunoreactive bands were visualized using enhanced chemoluminescence (ECL plus, Amersham Biosciences).

2.5. In situ dual staining on surgical forceps

To assess the applicability of our dual staining procedure in a clinical context, surgical forceps were cleaned following the same protocol as that applied to stainless steel tokens, and control images were taken to assess cleanliness and general state of the instrument. The forceps were then contaminated by immersing the tip into 10% ME7-infected brain homogenate (without Tween 20), with a contact time of less than a second, to simulate potential iatrogenic contamination of the instrument's surface. After letting the instrument dry for 30 min, dual staining was applied following the exact same protocol as described above. EDIC/EF images of ThT- and SR-positive signal were taken at 100× and 1000× magnification.

2.6. Statistical analysis

Various mixtures of ME7-infected brain homogenate in NBH were tested on different days and each mixture was tested approximately 10 times. The average amount of positive thioflavin T signal observed for each mixture was plotted against the theoretical ME7 content (percentage of ME7-infected brain in the mix). Following ANOVA, a *t*-test was employed to compare the signal of thioflavin T for each ME7 concentration against the NBH reference, and correlation between the thioflavin T signal and the proportion of ME7-infected homogenate was assessed using the Spearman's rank test in the SPSS package. The amount of thioflavin-positive material observed in various areas from hamster tissues was compared (*t*-test) with that of corresponding negative controls. Values of $P \leq 0.05$ were considered significant.

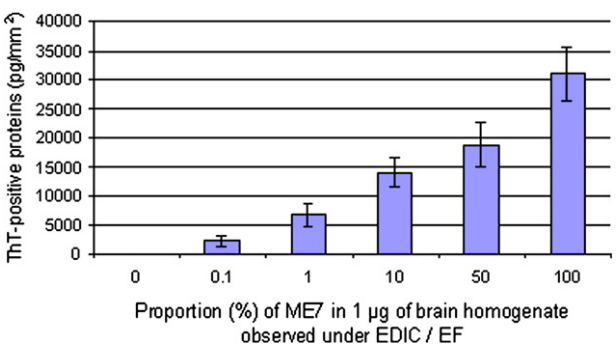

Fig. 2. Thioflavin T signal (482 nm) in anonymized samples containing various proportions of ME7-infected brain homogenate. Signal from NBH was set as threshold and fluorescence measured as pixels per total area observed (10 areas were observed for each sample) was expressed as pg/mm^2. Data are mean ± SD of ten separate experiments.

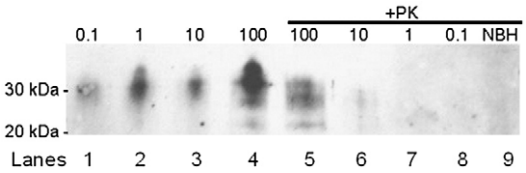

Fig. 3. Comparison of the limit of detection of the same amount of material as that observed directly under EDIC/EF (Figs. 1 and 2) by classic western blotting. PrP in various concentrations of ME7-infected brain homogenate prepared into normal brain homogenate (NBH; down to 0.1% v/v) could be detected prior to Proteinase K (PK) digestion (lanes 1–4). Following partial digestion with PK (30 min), the signal from a 1 µg mix containing 1% or less ME7-infected homogenate in NBH (lanes 7–8) was not distinguishable from 1 µg NBH alone (lane 9).

3. Results

3.1. Addition of Tween 20 improves recovery and homogenisation of small amounts of amyloid-infected tissues, facilitating sampling and observation and improving detection sensitivity

All 10% mice brain homogenates used for this study had a protein concentration of approximately 16 mg ml^{-1}. Our working protein concentration was normalised to 1 mg ml^{-1}, i.e. at least 1/16 dilutions of the original "10%" material. In preliminary experiments we tried to achieve a dose-related signal to calibrate the assay and determine the lowest limit of discrimination of amyloid in tissues, using dilutions of ME7-infected brain in NBH, while maintaining the same final protein concentration of 1 mg ml^{-1}. Following analysis, the thioflavin signal reported by volunteers appeared more elevated in ME7-containing mixes as expected, though the amount detected in intermediate dilutions did not correlate with the theoretical amount of PrP^{Sc} present in the mixture following serial 1/10 dilutions of ME7-infected brain in NBH. In addition, the total amount of tissues observable apparently varied between different mixes, with relatively large aggregates in some fields of view and almost no tissues in others. These discrepancies appeared due to tissue adsorption on the surface of plastic containers used to prepare the samples and aggregation of homogenates, which might have been accentuated by the drying on the stainless steel (Fig. 1a–c). For the purpose of this series of experiments we added Tween 20 to a final concentration of 0.1% (v/v) in all our normalized brain homogenates prior to distributing and mixing in anonymised tubes. This resulted in a more homogenous tissue distribution (Fig. 1, d–o), reduced the number and size of aggregates responsible for elevated background staining, and eventually led to a better correlation between the ThT signal and the theoretical PrP^{Sc} concentration in the samples (Fig. 2). Since the initial discrepancies reported with the thioflavin staining were associated with the presence of large tissue aggregates, observers were asked to exclude any remaining aggregate from subsequent observation and analysis. In the presence of Tween 20, the total amount of protein available for observation did not vary significantly between the different samples, as assessed by the SR scan of the whole sample (data not shown). Protein deposition also appeared under the form of smaller and more evenly spread particles producing little or no background, allowing for a better contrasted characteristic fluorescence of ThT bound to

amyloid, where present. Interestingly, amyloid-positive particles appeared mostly separated from other proteinaceous aggregates, suggesting that there was no accumulation of other proteins around these amyloid cores.

In all samples containing at least 0.1% ME7-infected brain homogenate, the mean ThT fluorescence (at 482 nm) observed in ten different fields without aggregates and corrected for background for image analysis, was significantly higher than that of NBH only (Fig. 2; $P<0.01$). Taking into account that (1) the ME7-infected and control "10% brain homogenates", appellation based on total weight, were diluted approximately 16-fold to obtain the working protein concentration of 1 mg ml^{-1} (as assessed by protein assay), and (2) volunteers detected amyloid-positive material in 3-log dilutions of ME7-infected brain into NBH (the "0.1% mixture"), we can conclude that we achieved rapid visual detection of characteristic amyloid aggregates from 1 µg of infected brain homogenate diluted 1/16000 (1/16 dilution in deionized water followed by 1/1000 dilution in NBH at the same protein concentration), i.e. 10-100 pg of infected tissue. Furthermore, volunteers sampled only ten representative areas at 1000× magnification for image analysis, which corresponded to a small fraction of the whole sample, therefore we can estimate the detection level of this dual staining method to be in the nanogram range, without the need of intermediate amplification steps. Some of the anonymised samples were NBH only to assess the risk of false positives. Reported levels of ThT signal in 0.01% ME7-infected brain mixtures were undistinguishable from the background signal observed in negative controls using our image analysis software (data not shown). When PrP^{Sc} could be detected and measured, we obtained a positive correlation (Spearman's rho = 0.896) between the ThT signal and the theoretical concentration of PrP^{Sc} in the samples.

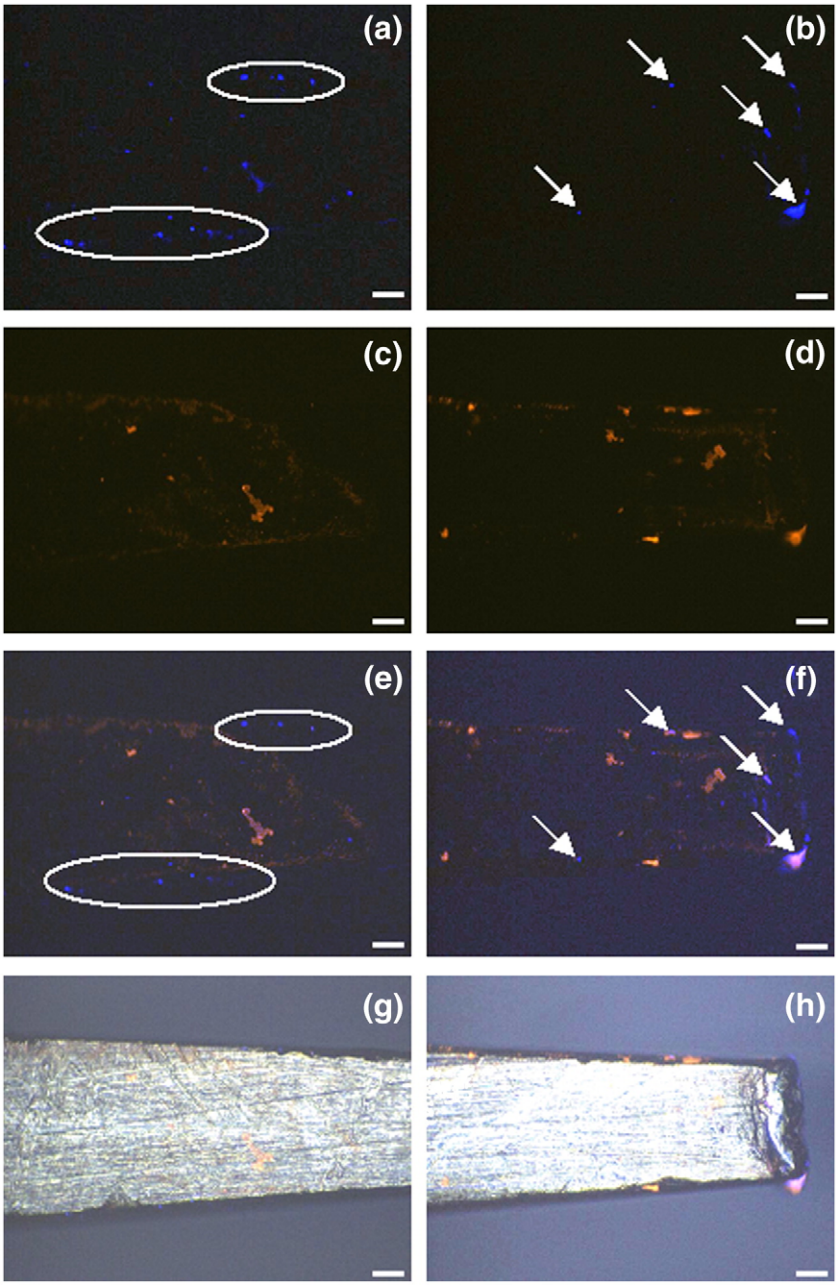

Fig. 4. Epifluorescence micrographs of dual stained ME7-infected brain homogenate contamination on two different areas of surgical forceps; (a and b) SYPRO Ruby staining, (c and d) thioflavin T staining, (e and f) merged pictures of both staining, (g and h) pictures of staining combined with EDIC white light image of the instrument. Bars: 100 µm.

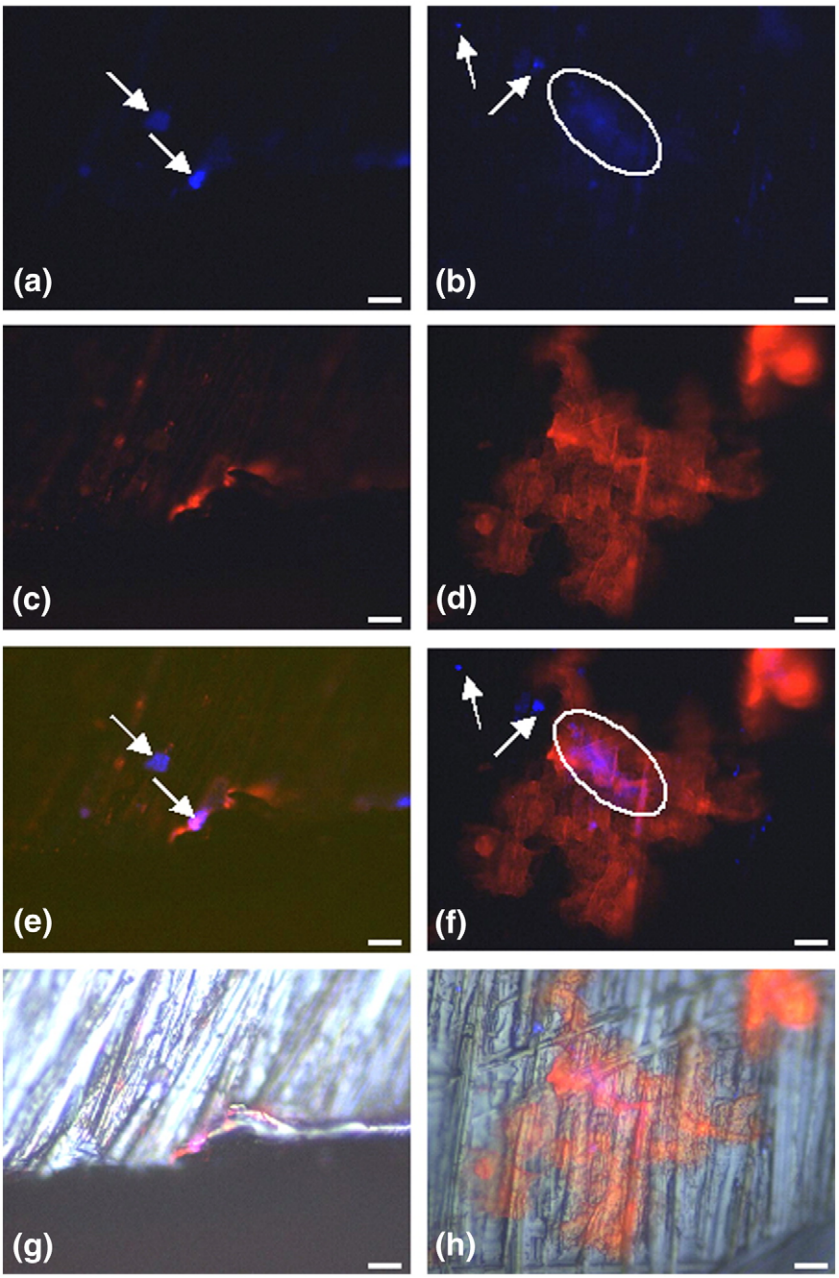

Fig. 5. High magnification epifluorescence micrographs of dual stained ME7-infected brain homogenate contamination on two different areas of surgical forceps; (a and b) SYPRO Ruby staining, (c and d) thioflavin T staining, (e and f) merged pictures of both staining, (g and h) pictures of staining combined with EDIC white light image of the instrument. Bars: 10 µm.

3.2. Sensitivity of the dual staining method remains at least 2-log higher than that of Western blot using the same samples

PrP was detected in all samples prior to partial PK digestion, using only 1 µg of tissue per lane (Fig. 3, lanes 1–4). Following partial digestion with PK, the samples containing ME7 only produced clear bands characteristic of PrP^{Sc} (Fig. 3, lane 5). The mixes containing 10% ME7 (v/v, equivalent to 0.1 µg of protein from ME7 brain) rarely produced visible bands (Fig. 3, lane 6), whereas the 1% or 0.1% ME7 mixes (v/v, equivalent to 10 ng of protein from ME7 brain) were never distinguishable from the NBH control (Fig. 3, lanes 7–9).

3.3. Detection of contamination on spiked surgical instruments

Biopsy forceps artificially contaminated and left to dry for 30 min, as might happen in a clinical setting, could be readily stained and contamination observed using EDIC/EF microscopy. After the forceps were put briefly (less than a second) in contact with ME7-infected brain homogenate, general contamination was observed at 100× magnification (Fig. 4). Interestingly, ThT-positive material could be distinguished from general protein contamination under 1000× magnification (Fig. 5). Protein contamination was mostly present in the indented areas of the instrument, as well as in the grooves naturally present on the stainless steel surface. On average 47.2 µg of tissue protein and 642.6 pg of PrP^{Sc} amyloid had bound per mm^2 of the instrument surface, even in less than one second contact time.

4. Discussion

4.1. The preparation and handling of samples is critical for the detection of amyloid aggregates in samples containing low amounts of PrP^{Sc}

Most samples of brain homogenates are prepared in PBS and stored in plastic containers, which might be detrimental to the

detection of low levels of PrP^{Sc} due to its hydrophobic nature and tendency to adhere strongly to surfaces. This renders detection and precise quantification of amyloid content difficult in samples containing low amounts of infected material, and only the highest concentrations (greater than 50% mixes, i.e. containing at least 0.5 µg of ME7-infected material) were clearly "positive" in our observations (data not shown). In addition, the aggregation of tissue is likely to vary between different species and different tissues, and this is an inherent risk of misidentification. Other studies have reported the influence of the interactions between lipids and PrP in terms of both neurotoxicity (Wang et al., 2006) and conformation changes (Wang et al., 2007). Interestingly, Deleault et al. (2007) were able to synthesize *de novo* infectious PrP^{Sc} molecules by protein misfolding cyclic amplification, using simply native PrP^{C} molecules in the presence of copurified lipid molecules and a synthetic polyanion. Interactions between prion amyloid and tissue lipids in PBS-diluted homogenates are likely to affect the detection of PrP in its different conformations from lipid-rich tissues such as brain. The addition of Tween 20 to all homogenate samples prepared for the calibration improved the recovery of the prion-containing fractions of brain homogenate from plastic Eppendorf tubes and pipette tips during the preparation of serial dilutions, as assessed by the clearer ThT and SR signals under the microscope for the same volumes of samples. This might be of relevance to other methods involving the preparation and storage of small volumes of PrP^{Sc} sample in plastic containers and their subsequent use in quantitative assays.

We found that ThT was prone to produce a relatively high background signal in aggregates of tissue on heavily soiled surfaces, albeit at a lower emission wavelength than for prion amyloid plaques. Although the addition of Tween 20 did not totally prevent aggregation to some degree, the improved homogeneity and the selection of aggregate-free areas for analysis allowed eliminating virtually all elevated background created by relatively thick tissue lumps left in the samples. This stronger signal in aggregates was observed for all wavelengths used, and the SR staining discriminated and helped to confirm when an apparently strong ThT signal was in fact higher background created by thicker aggregates of tissues in the homogenate.

Despite the difficulties inherent to the use of ThT reported elsewhere (Nilsson, 2004) and the very low amounts of impure material examined with the present technique, we observed a positive correlation between the ThT signal and the expected relative amount of PrP^{Sc} in these specifically prepared samples. Double-blind studies produced a dose-related signal on contaminated surgical stainless steel surfaces down to 3-log dilutions of ME7-infected brain into normal homogenates, as assessed by image analysis. Our level of detection using Western blot was similar to what we reported before (Lipscomb et al., 2007) or those reported elsewhere (Wadsworth et al., 2001; Beekes et al., 1995) and equates to at least a 2-log lower sensitivity of detection compared to the ThT/SR dual stain procedure described above, when applied to the same amount of material (1 µg total protein).

Sensitive Western blotting assays using sodium phosphotungstic acid precipitation have been reported to allow the detection of PrP^{Sc} from 5 nl of 10% vCJD brain homogenate (Wadsworth et al., 2001). This equates to 500 ng of tissue prior to PK digestion. In accordance with these data, in our studies PrP^{Sc} could not be detected by Western blot beyond a 1/10 dilution of 1 µg of 10% ME7-infected brain diluted in normal brain homogenate (i.e. 100 ng of ME7-infected homogenate). In contrast, the dual staining procedure allowed detection down to less than 1 ng of ME7-infected homogenate. Interestingly, the fact that amyloid-positive material appeared clearly dissociated from other proteinaceous deposition in all prepared dilutions confirms that the dual staining procedure is clearly discriminating between amyloid and general tissue proteins.

As we discussed previously when reporting results using thiazole reagents alone (Lipscomb et al., 2007), it has to be noted that ThT is capable of detecting various forms of amyloid aggregations, which therefore does not preclude its application to amyloidogenic prion diseases, but also potentially includes Alzheimer's disease and other amyloidoses. ThT has also been successfully used to detect amyloid adhesins in bacterial biofilms (Larsen et al., 2007) and in more complex mixtures such as sludge flocs from wastewater treatment plants (Larsen et al., 2008). The presence of such bacterial components in tissues is unlikely, but bacterial biofilms are not uncommon on the surface of some surgical instruments, or inside the lumen of endoscopes. The present method has potential for pathological studies, and also routine surveillance of tissues or surgical instruments (see below). It does not allow the identification of a particular amyloidosis or prion strain, as other methods might do. However other methods such as Western blot require more material to produce a clear result, therefore more complex intermediate steps such as protein misfolding cyclic amplification (PMCA; Saborio et al., 2001) are required. Our method trades strain specificity for sensitivity, low cost of consumables involved, ease of use and rapidity.

4.2. This technique offers great potential to rapidly assess the decontamination of surgical instruments, with quantitative discrimination between amyloid and other protein deposits

Our direct observation technique allows us to observe the inside of grooves and other areas on a surgical instrument, otherwise difficult to swab. The sensitivity of our method is likely to be even greater than demonstrated here since testing instrument surfaces by Western blot relies on swabbing the contaminated surface, and PrP^{Sc} is known to bind strongly to surfaces. Here we have not tried to elute or swab the 1 µl contamination to assess how much could be recovered for Western blot, however our experience is that amyloid is difficult to remove from the surfaces it has attached upon, and even more difficult to transfer back integrally into another container or onto another support.

As we reported before using a single ThT staining procedure (Lipscomb et al., 2007), the new ThT/SR dual staining procedure allowed visualisation and discrimination of very low levels of amyloid and other proteinaceous contamination on surgical instruments. Worryingly, measurements from the whole instrument converted to mass (Lipscomb et al., 2006b) revealed approximately 47.2 µg of tissue protein and 642.6 pg of PrP^{Sc} amyloid per mm^2 of the instrument surface after only one second contact time. This level of amyloid contamination equates to 18 femtomoles of PrP^{Sc} per mm^2 and is of concern since this could represent many infectious doses. Clearly, even brief surgical procedures on infected patients may lead to the binding of prion amyloid to instrument surfaces, and present a risk of iatrogenic transmission if the instruments are not successfully cleaned. Both general tissue protein and prion amyloid contamination tended to aggregate in grooves or pits present on the surface of the instrument, which would make their removal more difficult. Although no Tween 20 was added for these experiments, thioflavin-positive deposits could be clearly distinguished from other proteins. These results suggest that general tissue proteins do not tend to aggregate around amyloid cores. Alternatively, some amyloid material could be masked by the accumulation of non-amyloid proteins around an amyloid core, preventing the binding of ThT. However, no large aggregates were found to adhere on the surface of the instruments, since these tended to be washed away during simple rinsing procedures, and only small particles remained in grooves and pits. Further studies shall investigate how the concentration of amyloid and other molecular constituents influence the binding capacity of the tissue. Similarly, this technique offers great potential to rapidly assess cleaning chemistries and methods of decontamination of surgical instruments *in situ,* without introducing less sensitive swabbing or removal artefacts.

It has been reported that amyloid accumulation can be observed in aging individuals, independently of any pathology (Rowe et al., 2007). A baseline of "normal amyloid content" might need to be established

for such sensitive methods, which can be achieved by numerous observations of non-diseased brain, and potentially other tissues. The thiazole dyes do not discriminate between amyloid plaques composed of PrP^{Sc} 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.

We tried to establish a standard curve using recombinant PrP in the same conditions, but the purified material we used did not adhere sufficiently on the stainless steel surface and was mostly washed out, thus only a few particles were observed (data not showed). Fortunately, the present method can be used to quantify the concentration of PrP^{Sc} amyloid directly in infected brain and other tissues without resorting to reference standards. Those recombinant molecules are often not identical to the protein in its native state in diseased tissues, and therefore might present different detectability characteristics.

5. Conclusion

This new sensitive microscopy procedure is rapid and can be applied directly to instrument surfaces to discriminate and measure amyloid and general tissue proteins contamination. As such, it offers a simple way to assess current protocols for instrument decontamination in washer/disinfector systems of sterile service departments, which should contribute to reducing the risk of iatrogenic transmission of CJD and other proteinaceous infectious agents. ThT/SR dual staining may also be used as such as a quantitative analysis tool to detect abnormal levels of amyloid aggregation in small samples of tissues and may offer an alternative method for rapid screening of biopsy samples.

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