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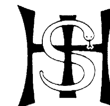

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Rapid method for the sensitive detection of protein contamination on surgical instruments

I.P. Lipscomb*, A.K. Sihota, M. Botham, K.L. Harris, C.W. Keevil

Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Southampton, UK

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Summary Hospital sterile service departments (SSDs) currently rely on simple visual confirmation of cleanliness as an assessment of the efficacy of cleaning surgical instruments. The inherent inability to monitor low levels of infectious or proteinaceous contamination on surgical instruments creates the possibility that highly dangerous and robust biological agents may remain infectious and undetected even after standard cleaning and sterilization procedures have been employed. This paper describes the development of a novel microscopy technique, episcopic differential interference contrast microscope, combined with the fluorescent reagent, SYPRO Ruby, to rapidly detect brain tissue protein to below 400 pg/mm^2 on an instrument surface. This technique has displayed a minimum level of detection observed by 50% of volunteers of 85 pg/mm^2 (95% confidence intervals 67–112 pg/mm^2). Quantitative assessment of instruments supplied from various SSDs enabled the establishment of a 'contamination index' of both proteinaceous and non-proteinaceous deposits on the surface. This new methodology for the assessment of surface contamination is generally applicable and should facilitate future quantitative surveys of instrument contamination in hospitals and other healthcare environments.
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Introduction

It is estimated that there are over two million cases of hospital-acquired infection in the USA each year, and these incidents are thought to cause around 88 000 deaths per annum.[1] This figure creates a substantial socio-economic burden for the health service, with the extra costs incurred in the USA considered to be in excess of $5 billion.[1] Although a large number of these cases, approximately 30%, are thought to be preventable,[2] the requirement to produce clean instruments is an 'essential

* Corresponding author. Tel.: +44 238 059 2034; fax: +44 235 94459.
E-mail address: i.lipscomb@soton.ac.uk

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prerequisite' to ensure effective disinfectant or sterilant activity.[3]

In recent years, evidence has emerged for a highly robust, infection-related, fatal, proteinaceous agent termed 'prion' (PrP^{sc}) found in spongiform encephalopathies, such as variant Creutzfeldt-Jakob disease[4] or chronic wasting disease.[5] The prion alters its secondary structure from largely α-helical (PrP^{c}) to predominantly β-sheet (PrP^{sc}), and forms amyloid plaques. These can resist traditional cleaning and sterilization strategies employed by modern sterile service departments (SSDs),[6] and their ability to infect iatrogenically through contaminated medical devices has been well documented.[7] Recently, the sensitivity of the current recommended protein test, ninhydrin,[8] has been brought into question,[9] and this concern has further increased the requirement for a sensitive means of assessing protein contamination of surgical instruments, especially for those instruments deemed to be 'high risk' such as neurosurgical items.[10]

Current clinically applied methods for the detection of proteins or infectious organisms on surfaces largely depend on protocols that involve the sampling of the area of concern, with a swab[11] or wipe,[12] and the subsequent ex situ detection of any contamination on the collection material. This detection may involve traditional tissue culture[13] techniques, or the application of other methods such as protein chemistry, enzyme immunoassay[14] or quantitative polymerase chain reaction.[15] However, it is readily apparent that such approaches are inefficient, expensive and are unlikely to detect low levels of contamination. The detection of low levels of protein contamination on the highly complex surfaces of surgical instruments, varying from forceps to endoscopes, poses particular problems. Polished or mirrored surfaces are not perfectly flat and tend to become increasingly pitted or scratched with prolonged use. This makes complete removal of protein from the numerous binding sites, including the indentations, difficult.

More sophisticated detection techniques such as surface plasmon resonance (SPR),[16] X-ray photoelectron spectroscopy (XPS),[17] and time of flight secondary ion mass spectrometry (ToF-SIMS)[17] have been shown to be sensitive and effective at distinguishing small amounts of bound proteinaceous soiling on selected substrates (SPR = 15 pg/mm^2, XPS = 100 pg/mm^2, ToF-SIMS = 1 pg/mm^2).[17,18] However such systems are too expensive, complex and slow to be able to handle both the size and complexity of some instruments as well as the high throughputs required to make them applicable within an SSD.

Commercial surface hygiene tests have been available in the food industry for several years but are again based on either swabbing/wiping techniques, or the utilization of specific chemical interactions, both of which can have drawbacks in certain situations. More direct staining using laboratory-based protein dyes that are capable of performing this monitoring function tend to be too insensitive or too expensive to warrant application, e.g. ninhydrin.[19]

Consequently, a new method is required for the detection of protein contamination on surgical instruments and other surfaces; the method for detection must be simple, rapid, robust and easily reproducible, and must avoid possible contamination of other detection instruments yet retain high sensitivity. We have taken advantage of new developments in light microscopy and used episcopic differential interference contrast/epifluorescence (EDIC/EF) techniques[20] and a sensitive fluorescent dye, SYPRO Ruby,[21] for the detection of very low levels of protein contamination on surgical metal surfaces. This has enabled the development of a contamination index for the assessment of instruments supplied by hospital SSDs.

Methods

Tissue and protein controls

Brain tissue was used as a source of complex tissue for studies of neurosurgical contamination. Female C57BL/6J mice were killed and the brains were removed, frozen in liquid N_2 vapour and stored at -80°C. Brains were weighed before use and a 10% (w/v) homogenate was made in distilled water. Protein concentrations of serial dilutions of the brain homogenate were assessed using a total protein assay (Bio-rad, Hercules, CA, USA).

Proteinaceous contamination

Low levels of proteinaceous deposits were detected using SYPRO Ruby (Molecular Probes, Eugene, OR, USA). This is a ruthenium-based fluorescent stain that reacts non-covalently with proteins. The stained proteinaceous material can be excited by ultraviolet light of around 302 nm or visible light of about 470 nm, and produces an emission signal around 618 nm. These properties correspond to the fluorescence filter set fitted in the EDIC microscope. The sensitivity of SYPRO Ruby in agarose gels is usually cited as being in the sub-nanogram/mm^2 range.[21] However, it is not known whether the same

sensitivity might be expected in a situation where the protein is bound to a metal surface. The application of SYPRO Ruby dye as a protein stain for proteins absorbed on to metal substrata has never been reported.

Brain homogenate dilutions were applied to stainless steel coupons. The coupons (25 mm × 75 mm) were made from 316L-grade stainless steel and had previously passed through a two-step cleaning process to produce a 'pristine' surface free from any residual soiling (Table I). The cleaning process initially involved washing the tokens in acetone for 10 min to remove grease and lipid deposits. The tokens were then rinsed in filtered distilled water for 2 × 10 min and then placed in a dry heat furnace (220 °C) for a minimum of 15 h. The tokens were subsequently removed and allowed to cool to room temperature before any staining procedure was initiated.

Diluted tissue homogenate samples of 1 μL were pipetted on to the stainless steel coupons, and these were incubated at 37 °C for 30 min. Replicate samples were stained with SYPRO Ruby for 15 min and washed in filtered (0.2-μm membrane) distilled water for 10 min before visualization with EDIC/EF microscopy.

To assess the sensitivity of protein or general contamination detection using EDIC microscopy and SYPRO Ruby, eight independent observers were required to score the SYPRO Ruby signal as present or absent in 12 replicates of anonymous sample concentrations. In addition, representative digital images were captured at ×400 magnification for dilutions of brain homogenate on steel coupons ($N = 4$), and the per area signal response (the field of view quantified was 0.1 mm^2) was calculated in relation to the protein concentration applied.

Surgical instruments

Twenty-three instruments were obtained from an anonymous hospital trust and assessed for contamination. The surgical instruments ranged in size and shape. All had passed through traditional machine washer-disinfector cleaning procedures and had been deemed to be clean. The instruments were assessed by two methods; a visual inspection and a microscopic examination after staining.

Prior to staining and microscope evaluation, the instruments were visually inspected for contamination or blemishes and were given a score dependent on the degree of contamination: 0 for no visible soiling, 1 for small amounts, and 2 for large amounts of visible soiling. The instruments were stained by the application of SYPRO Ruby for 15 min followed by washing three times for 5 min in filtered distilled water.

The 23 instruments were examined using the EDIC/EF microscope at multiple points over their surface. Thirty areas that displayed distinctive representative contamination based on the size of particulates and the percentage surface coverage observed in the microscope's field of view were photographed. A contamination index (0–4) was then established from these reference areas. Representative photomicrographs were taken of the different levels of the index, and these were placed in full view from where the microscope operator was positioned. Test instruments were then stained as described above and visualized under the microscope. The operator assessed defined sample areas by comparing the contamination viewed with the representative images, and allocated a score accordingly. This index enabled rapid assessment of the degree of contamination apparent on each region of interest, and multiregional sampling was performed on all instruments.

Microscopy

The EDIC/EF microscope employed for this study was specially designed with the analyser, polarizer and Nomarski prism incorporated into the head

Table I The defined parameters and equivalent protein concentrations for the contamination index

Contamination index	Particulate height (μm)	Particulate width (μm)	FOV coverage (%)	Protein/mm^2
1	0-5	0-3	1-2	0-42 ng
2	2-10	3-10	5-10	42-420 ng
3	5-20	10-50	20-50	0.42-4.2 μg
4	20-100	>50	>50	>4.2 μg
4a	20-100	>50	>50	>4.2 μg PE

FOV, microscope's field of view (0.36 mm^2); PE, protein equivalent soil that did not stain with SYPRO Ruby. Protein/mm^2 was calculated using the information that a 1-μm-diameter area of protein with an average molecular weight of 30 kDa and 3 μm in height has been calculated to be approximately equivalent to 1 pg (data not shown).

piece of the microscope.[22] This allows episcopic illumination to produce differential interference contrast (DIC)[23,24] images, and as such enables the system to rapidly and sensitively visualize opaque or semi-opaque surfaces. Long working distance lenses allow highly convoluted and complex structures to be visualized without coverslips or oil, and remove the possibility of cross-contamination from infectious specimens. This novel system combines biological, optical and materials science knowledge to produce a unique and highly sensitive system with the ability to detect low levels of both proteinaceous and non-proteinaceous contamination on surgical instrument surfaces. To produce quantified data, the imaged reference areas were analysed using Image Pro software (MediaCybernetics, Silver Spring, MD, USA).

Results

Proteinaceous contamination

The sensitivity of the protein detection method using SYPRO Ruby staining and EDIC/EF microscopy was tested on stainless steel surfaces with dilutions of brain homogenate. Eight independent observers visualized 12 replicate samples and were asked to indicate the number of positive signals present. A protein threshold value of 400 pg/mm^2 was visualized by all observers ($N=8$ observers, 12 replicates). By assessing the data from the dilution series, the values for the minimum level of detection (MLD) to be assured of a set percentage of positive results

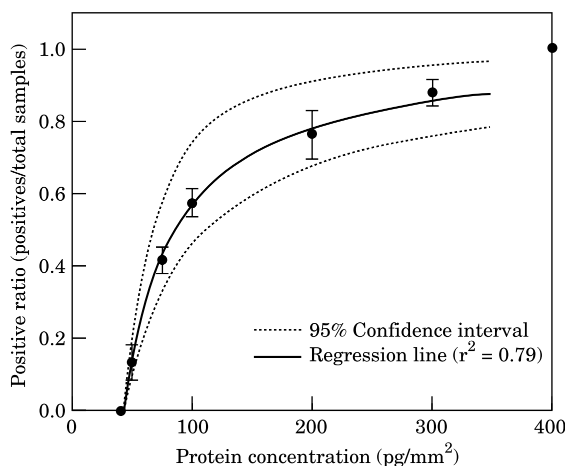

Figure 1 Results of dilution series showing where results were positive for brain homogenate ($N=8$ observers, 12 replicates per observer) on stainless steel surfaces. The minimum level of detection observed by 50% of volunteers was 85 pg/mm^2 (95% confidence intervals 67–112 pg/mm^2).

was calculated (Figure 1). This resulted in an MLD_{75} (detectable by 75% of observers) value of 175 pg/mm^2 [95% confidence intervals (CI) 104–286 pg/mm^2] and an MLD_{50} (detectable by 50% of observers) of 85 pg/mm^2 (95% CI 67–112 pg/mm^2). This finding was comparable with the degree of sensitivity (0.25–1 ng/mm^2) shown by previous investigations for proteins separated on polyacrylamide gels.[21]

The relationship between protein concentration and per area signal response (i.e. deemed by some volunteers to exhibit SYPRO Ruby staining) was investigated by comparing digitized images of the stained region. A linear relationship for protein concentrations of between 300 pg and 70 ng ($r^2=0.96$) was observed (data not shown).

Surgical instruments

The protocol developed for the sensitive detection of proteinaceous and non-proteinaceous material on surgical stainless steel was subsequently applied to instruments obtained from SSDs. A contamination index was established describing contaminant particle size, field of view coverage and protein/mm^2. Class 0 describes no detectable protein contamination while class 4 can exceed 21 μg protein/mm^2 covering over 50% of the observed instrument surface.

Although the degree and intensity of contamination varied, all instruments ($N=23$) examined showed signs of contamination on at least one of the sample regions. The scores were averaged for each instrument (Figure 2). Results indicated that over half (56%) of the instruments inspected showed severe (class 3–4) contamination in at least one of the sample regions, 35% were moderately contaminated (class 3), and only 9% displayed low-level deposition (class 0–2). The overall mean contamination index value for all the instruments was 2.8.

We also looked in more detail at the scores obtained from hinged items, such as forceps and scissors ($N=5$), as these have been identified as items that may harbour large reservoirs of unwanted material. If compared over the whole hinged subpopulation with the exception of the blade region, there is good correlation between visual inspection and microscopic analysis.

However, when instruments visually assessed as being low scoring, i.e. little apparent contamination (average surface score of <1), were observed using the EDIC/EF microscopy procedures, a large variation between the two methodologies became apparent in the majority of regions. Only the inner hinge region showed any degree of agreement between the two methodologies. The

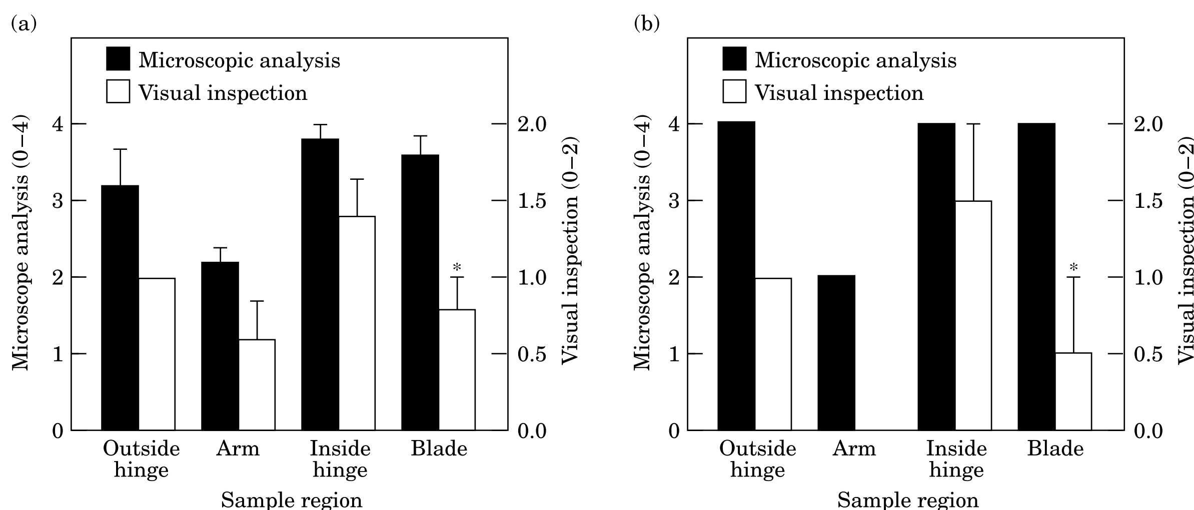

Figure 2 Regional variation in levels of contamination on (a) all hinged surgical instruments ($N=5$) and (b) those with low visual scores, and differences in findings between visual inspection (visual score: 0, no contamination; 2, heavily contaminated) and the microscopic inspection technique (contamination index score: 0, no contamination; 4, heavily contaminated).*, This area is more difficult to visually discern contamination due to the complexity and colouring of its surface.

average microscope analysis score for these instruments was 3.5. Clearly the EDIC/EF analysis procedure is much more sensitive than visual observation.

Discussion

Approximately 6.5 million surgical procedures are performed in England each year.[25] These procedures are spread across the 182 acute NHS trusts that cover the 249 hospitals with SSDs in England.[26] The emergence of evidence that highly robust pathogens such as the prion protein, a characteristic of variant or sporadic Creuztfeldt-Jakob disease, may remain viable following standard hospital decontamination procedures[6,27-29] led the Department of Health to issue revised guidelines on the decontamination of instruments (HSC 178_1999 and 179_1999) in August 1999.[30,31] However, it is clear that subsequent and ongoing monitoring of cleaning standards must be maintained in order to ensure that the highest decontamination standards are reached and maintained, and as such reduce any possibility of nosocomial infection.

Current methods of detecting contamination on instruments involve procedures that attempt to remove the contaminant such as swabbing or wiping, followed by protein or enzyme-linked immunosorbent assay detection of the sample. Such methods are insensitive, unreliable and prone to artefacts. Of these methods of surface hygiene monitoring, swabbing is the most common.[32] However, reports into its ability in taking effective samples vary significantly; for example, the recovery of surface contamination by the application of a single wet swab technique has been described to be as low as 19% by Angelotti *et al.*[33] Yamayoshi *et al.*[11] indicated better recovery rates of around 60% and Buttner *et al.*[15] described recovery rates of around 70%. However, in 2002, Sanderson *et al.*[34] reported a much lower value of around 54%. Wipe methods have been shown to produce higher recovery rates; however, even these have shown a large recovery variation of between 74% and 87% in spore experiments.[15,34] From these reports, it can clearly be seen that with such wide variation of surface contamination extraction values, any method based purely on a swab or wipe sampling should be treated with some degree of caution.

Commercial surface hygiene kits that detect protein have been available in the food industry for several years. These kits, such as the Biotrace Pro-tect® (Bridgend, UK) and DiverseyLever Check-PRO (Sturtevant, WI, USA),[35] are colourimetric in nature and rely on wiping or swabbing of a surface to elute the sample followed by a subsequent 'pass/fail' colour change. The commercial tests have been reported to achieve sensitivity levels of between 2 and 10 ng/mm^2.[36]

Ideally, a method should have no requirement for a sample to be removed from the surface in question but should visualize any contamination in situ. Such a method overcomes many problems of poor recovery, including retention of the contamination in pits or cracks on an instrument or surface.

One such surface hygiene system is that of ATP bioluminescence, which has grown in popularity over recent years and has a reputation as a sensitive method for the assessment of surface hygiene.[37] Adenosine tri-phosphate is widely found in organic debris and micro-organisms, and reacts with firefly luciferase to produce a light signal intensity that is directly equivalent in brightness to the amount of ATP and, hence, contamination present. However, ATP bioluminescence has been shown to be dramatically affected by both pH, temperature and the presence of industrial cleaning solutions.[37,38] In addition, this enzymatic indicator does not[35] detect contamination in situations such as protein-only soiling, where there are no active metabolizing cells and a low microbial count. As such, in a situation where the infectious agent is thought to be composed in full or in part by a protein, i.e. disease-causing prion (PrP^{sc}), it is evident that this test may give false confidence in the cleaning process applied.

Other in situ, protein-only stains have been widely used in the laboratory. These include colourimetric stains such as ninhydrin, Coomassie brilliant blue, Ponceau S, amido black and colloidal silver/gold. SYPRO Ruby has been shown to have far better sensitivity than the colourimetric stains and indeed reaches detection levels that rival colloidal gold.[21] This sensitivity was also displayed on the metal substrata-bound protein investigated in the current study. The stain itself is easy to apply and requires just 15 min to achieve maximum sensitivity, unlike the overnight procedures required for colloidal staining, and it has a reported capability to detect some peptides as small as seven amino acids.[39]

Although SYPRO Ruby will stain any protein and is not targeted at any specific type, it possesses a large Stoke's shift (excitation 450 nm, emission 618 nm). This ability creates a situation where any background non-proteinaceous material will emit light at around 500–550 nm under optimal illumination for SYPRO Ruby detection. As such, the positive signal, at 618 nm, is clearly visible and any background fluorescent signal can be ignored. The results gained from the surgical instruments and described in the current study appear to indicate that all the instruments had some contamination on their surface. However, the question must be raised whether a totally clean instrument is a desired goal. Park *et al.*[40] showed how pre-cleaning a metal surface with solvents led to an enhancement in the subsequent protein-loading density. So, it is not unreasonable to assume that a 'pristine' instrument may produce a better surface for protein/contamination absorption. If this instrument subsequently comes into contact with a highly infectious, yet robust agent, such as the prion agent, this increase in absorption sites could create a greater risk of iatrogenic transmission to other patients if subsequent decontamination procedures are ineffective. This situation is clearly highly undesirable and unacceptable. As such, the goal must be either to obtain complete effective cleaning all the time, a goal that is difficult to achieve, or to block contamination absorption through some form of protective covering or coating.

In this paper, we have demonstrated that it is possible to detect very low levels of protein contamination on surgical steel surfaces and instruments using EDIC/EF microscopy and fluorescent dyes. The simplicity of this approach lends itself to the further development of this methodology in a wide variety of settings. This versatility enables visualization of the surface of complex and convoluted opaque structures at high magnification without the requirement for conventional coverslips or oil immersion, and is easily quantifiable compared with the poor sampling and/or detection systems associated with traditional techniques such as surface swabbing and chemical or ATP assessment.

This investigation provides the first in situ description of proteinaceous contamination of surgical steel instruments. The methods employed have been shown to allow sensitive quantification of the contamination, and as such provide an important advance for the rapid assessment of potentially contaminated instruments. These initial results indicate the ineffectiveness of visual inspection as a monitoring tool and, although small in number, appear to indicate that considerable proteinaceous soiling is still present upon most of the instrument's surface even after cleaning.

Although the size of the prion infectious unit is undefined, the increased sensitivity of this work compared with traditional techniques may provide a major advance for public health and help to reduce iatrogenic transmission of robust pathogens such as the prion protein. As such, it can help to increase public confidence towards healthcare cleaning and decontamination procedures. Although the initial number of instruments inspected was very low, it is hoped that a larger number of instruments will be obtained for surveillance in the near future.

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