



The sensitivity of approved Ninhydrin and Biuret tests in the assessment of protein contamination on surgical steel as an aid to prevent iatrogenic prion transmission

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Summary Regulations recommend the routine application of biochemical tests, such as the Ninhydrin or Biuret tests, to confirm the efficacy of hospital sterile service department (SSD) washer-disinfector cycles in removing proteinaceous material, particularly with respect to prions. The effectiveness of these methods relies on both the effective sampling of the instruments and the sensitivity of the tests employed. Two commercially available contamination assessment tests were evaluated for their sensitivity to ME7 brain homogenate on surgical-grade stainless steel surfaces. Controls were visualized by the application of episcopic differential interference contrast Epi-fluorescence microscopy (EDICEF) combined with the sensitive fluorescent reagent, SYPRO Ruby, which has been shown previously to rapidly visualize and assess low levels of contamination on medical devices. The Ninhydrin test displayed a minimum level of detection observed by 75% of volunteers (MLD_{75}) of $9.25 \mu\text{g}$ [95% confidence interval (95% CI) $8.6\text{--}10.0 \mu\text{g}$]. The Biuret test provided better sensitivity, with a MLD_{75} of $6.7 \mu\text{g}$ (95% CI $5.4\text{--}8.2 \mu\text{g}$). However, much lower concentrations of proteinaceous soiling (pg) were visualized using the EDIC/EF microscopy method. From these findings, it is clear that these approved colorimetric tests of cleaning are relatively insensitive. This investigation demonstrates how large amounts (up to $6.5 \mu\text{g}$) of proteinaceous brain contamination could remain undetected and the instruments deemed clean using such

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methods. The application of more sensitive cleanliness evaluation methods should be applied to reduce the risk of iatrogenic transmission of prion disease in 'high-risk' instruments such as neurosurgical devices.

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Introduction

In 1999, a 'snapshot' survey of the decontamination services within the National Health Service (NHS) found that 109 of the 249 (44%) hospital sterile service departments (SSDs) in England did not meet acceptable decontamination standards.¹ Following this sample study, a comprehensive survey of the NHS in England was commenced in October 2000 and a health service circular was issued.² As a consequence of these reports, the UK Department of Health issued revised guidelines on the decontamination of instruments, and the British Government announced a £200 million investment programme to modernize all NHS decontamination/sterilization facilities.^{3–5}

Over 6.5 million operations are performed each year in England alone.⁶ These procedures produce approximately 9.2 million surgical trays that require decontamination.⁷ With an average of 12 instruments/set, this means that approximately 110 million instruments require decontamination each year, or in real terms, two million instruments/week spread over the 249 hospitals with SSDs.⁸ Therefore, the average SSD, which handles approximately 50 000 trays/year, processes in excess of 1500 instruments/day. When surgical instruments have been taken into an SSD, either after surgery or new, they are cleaned and disinfected in a mechanical washer-disinfector with the application of an enzymatic or alkaline detergent, which may include sonication. Once dry, the instruments tend to be inspected by visual methods and passed for any residual soiling or mechanical failure before being packaged and sent for sterilization. It has been recommended that visual inspections should be performed daily in order to verify the efficacy of cleaning.⁹

Although it has been reported that haem pigment levels of 10 µg/cm² can be detected, it has also been reported that bodily fluids without pigments are difficult to visualize, even in large quantities.^{9,10} This is an important parameter because, for example, human cerebral spinal fluid is both colourless and odourless but has been shown to be a carrier of infection of prion diseases; a group of neurodegenerative and invariably fatal

conditions that include variant Creutzfeldt-Jakob disease.¹¹

As such, the need for sensitive assessment measures that ensure the highest standards of cleanliness are maintained is paramount. European guidelines ISO EN15883 and the British Health Technical Memorandum (HTM) 2030 provide recommendations for the assessment of instrument cleanliness. Two of the techniques outlined in these documents are based on the Biuret and Ninhydrin chemical reactions, requiring prior swabbing of instrument surfaces before chromogenic detection. The Biuret technique employs the reaction of copper ions to form a complex with peptide bonds under alkaline conditions; i.e. in the presence of sodium hydroxide, such ions are reduced from Cu²⁺ to Cu⁺. The addition of bicinchoninic acid (BCA) causes these copper ions to react and form a purple complex that can be readily visualized.

The Biuret test kit (Pro-TECT M, Biotrace, Bridgend, UK) applied within this investigation consists of a swab and a tube containing both BCA and a copper sulphate solution separated by a thin film. After the test region has been swabbed, the swab is placed into the tube firmly, breaking the film thus allowing the two chemicals to mix and the Biuret reaction to start. The test requires incubation at 37 °C for 45 min to achieve the highest sensitivity.¹²

Application of the Ninhydrin reagent in the detection of latent fingerprints and the assessment of surface contamination is well documented.^{13–15}

The methodology is based on the reaction of amino acids, peptides and proteins with 1,2,3-indantrione monohydrate. Classical Ninhydrin testing involves swabbing of the test surface followed by application of the reagent to the swab before incubation for 30 min at 110 °C in a dry oven.¹⁶ The Ninhydrin kit (Albert Browne Ltd, Leicester, UK) tested in this investigation utilizes Ninhydrin gel, which has fewer complications than the classical reagent and requires incubation at a lower temperature of 57 °C for approximately 60 min.

The present study was carried out to investigate the ability of the ISO-EN-15883- and HTM-2030-approved colorimetric tests to detect low

levels of prion-infected brain material on surgical stainless steel surfaces.

Methods

Tissue

Prion-infected brain material was obtained from female C57BL/6J mice that had been injected with 1 μ L of 10% (w/v) ME7-infected brain homogenate into the dorsal hippocampal region of the brain, as described elsewhere.¹⁷ All animals were killed 19–21 weeks post inoculation. The tissue was frozen in liquid nitrogen, subsequent ME7 10% (w/v) brain homogenate was produced, and the protein concentrations were assessed by the application of a total protein assay (Bio-Rad, Hercules, USA).

Stainless steel tokens

The tokens (25 mm \times 75 mm) were made from 316L surgical-grade stainless steel and had passed through a previously described two-step cleaning process to produce a 'pristine' surface free from any residual soiling prior to artificial contamination.¹⁸

Soiling and testing

The tokens were soiled with 4- μ L drops of serial dilutions of ME7-prion brain homogenate; these were allowed to dry at 37 °C for 30 min before being assessed with the protein detection test kits.

Positive controls for all the dilutions were obtained by staining with SYPRO Ruby (Invitrogen, Paisley, UK) and observation using the episcopic differential interference contrast Epi-fluorescence (EDIC/EF) light microscopy method.¹⁸ All of the dilutions were clearly visible using this procedure. Negative controls were soiled with distilled water alone.

Swabs were moistened with four drops of distilled water and swabbing of the test area was carried out by rotating the swab, as per the manufacturer's guidelines.

For the Ninhydrin test (Albert Brown Ltd, Leicester, UK), the swabs were placed into the Ninhydrin gel vials provided and incubated at 57 °C for 60 min. The vials were then inspected and scored: 0, no colour (negative); 1, slight purple colour; and 2, dark purple colour.

For the Biuret test (Biotrace, Bridgend, UK), the swabs were pressed firmly into the test solution until it clicked, in accordance with the manufacturer's instructions. The test vials were incubated at 37 °C for 45 min, inspected and scored: 0, green (negative); 1, grey colour; 2, light purple colour; and 3, dark purple colour.

The tests were then repeated ($N=6$) and a mean resultant score was obtained for each of the serial dilutions.

Results

The prion-infected brain homogenate controls were placed on to stainless steel tokens and examined under the EDIC/EF microscope. The photomicrographs obtained show that low levels of proteinaceous contamination can be observed readily on the tokens, down to a level below 0.4 μ g (Figure 1).

The Ninhydrin protein assessment techniques were then applied to known samples with the results obtained from the test, indicating a minimum level of detection observed by 75% of volunteers (MLD₇₅) sensitivity level of 9.25 μ g [95% confidence interval (CI) 8.6–10.0 μ g] (Figure 2).

Similar results were obtained from the assessment of the Biuret test, with a resultant MLD₇₅ sensitivity level of 6.7 μ g (95% CI 5.4–8.2 μ g) being recorded (Figure 3).

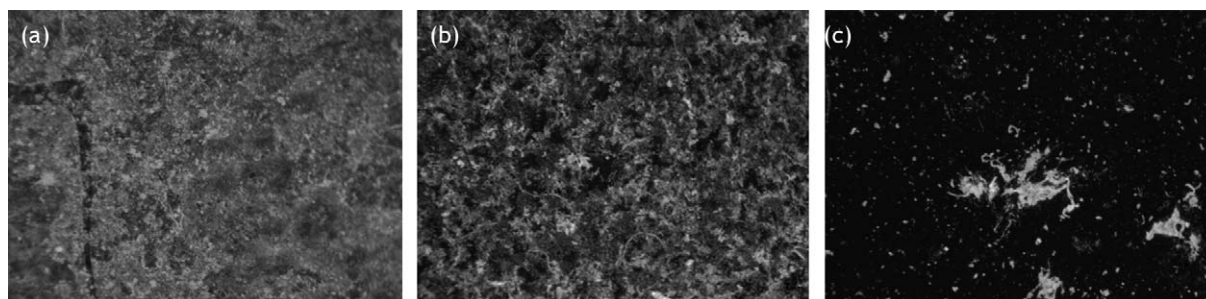


Figure 1 Photomicrographs of control tokens using episcopic differential interference contrast microscopy/epi-fluorescence in conjunction with SYPRO Ruby. The presence of homogenate can be visualized clearly at levels of (a) 10 μ g, (b) 4 μ g and (c) 0.4 μ g.

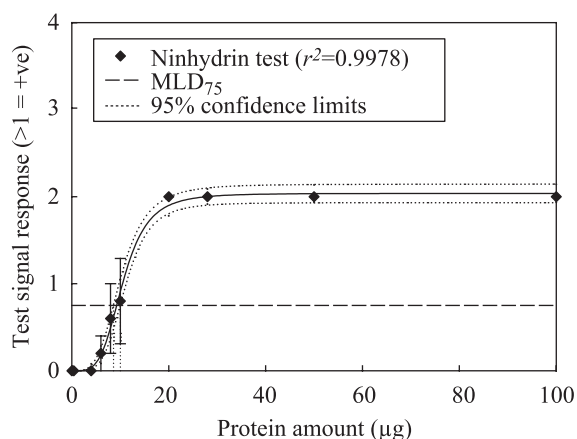


Figure 2 Ninhydrin test sensitivity results giving a minimum level of detection observed by 75% of volunteers (MLD_{75}) sensitivity level of 9.25 μg (95% confidence interval 8.6–10.0 μg).

Discussion

There are approximately 6.5 million surgical procedures performed within England each year.¹⁹ These procedures are spread across the 182 acute NHS trusts, which themselves cover the 249 hospitals with SSDs in England.²⁰ The emergence of evidence that highly robust infectious agents such as the prion protein, a characteristic of variant and sporadic Creutzfeldt-Jakob disease, may remain viable following standard hospital decontaminating procedures led the Department of Health to issue revised guidelines on the decontamination of instruments (HSC 178_1999 and 179_1999) in August 1999.^{3,4,11,21–23} However, it is clear that subsequent and ongoing monitoring of cleaning standards must be maintained in order to ensure that the highest decontamination

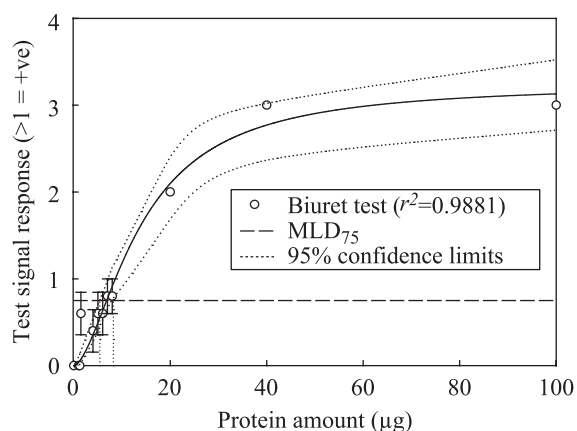


Figure 3 Biuret test sensitivity results giving a minimum level of detection observed by 75% of volunteers (MLD_{75}) sensitivity level of 6.7 μg (95% confidence interval 5.4–8.2 μg).

standards are reached and maintained, and thus reduce any possibility of nosocomial infection.

The efficacy of the cleaning process is traditionally assessed using visual inspection of the instruments. Time constraints and the sheer number of instruments involved must cast doubt on both the ability and the validity of visual inspection to ensure that effective cleaning is achieved. In 1995, a study found that although over 90% (29/32) of the visually inspected instruments looked clean, over 84% possessed some residual soiling.²⁴ This study did not attempt to identify the origin of the debris. Recommendations of both the European Union (ISO EN 15883) and the British Government (HTM 2030) outline the regular application of approved biochemical assessment methods to be performed on instruments in addition to the day-to-day visual inspection.

The recent emergence of highly robust infectious agents such as the prion protein that can resist conventional inactivation cycles has increased the need to validate cleaning procedures and ensure that instruments are indeed clean.²⁵ The present study was carried out to test two of the ISO-EN-15883- and HTM-2030-approved colorimetric tests (Ninhydrin and Biuret), and assessed their ability to detect low levels of prion-infected brain material on surgical stainless steel surfaces.

The findings from this investigation indicate that MLD_{75} values for the Ninhydrin test and the Biuret test were 9.25 and 6.7 μg , respectively. Assuming a minimum sample area of at least 1 cm^2 , this would equate to a sensitivity of detection of 9.25 and 6.7 $\mu\text{g}/\text{cm}^2$, respectively. Consider the average prion molecule to be approximately 30 kDa in mass, and 1 infectious unit to be 10^5 PrP^{Sc} molecules, then around 1×10^6 IU could be present before a positive, 'instrument dirty', result would occur; a situation which is clearly unacceptable.²⁶

The cause of this insensitivity, however, may not be directly related to the colorimetric tests themselves but more to the sampling method used. Swabbing has long been reported to produce unreliable surface soil recovery, and, in some instances, as little as 19% of the surface contamination was recovered.^{18,27} As such, any system that is based on this sampling technique must suffer from inherent inaccuracies. Indeed, this sensitivity problem may be compounded by the fact that actual surgical instruments can remain in service for over 10 years, and although there is little work published in this area, it is logical to assume that this continued use is likely to age an instrument and hence increase the amount of pitting and scratches present on its surface. Such markings will consequently add to the overall surface roughness and, in turn, increase

the number of sites to which soil can adhere. It is also worth noting that a large number of surgical instruments such as tissue forceps possess both serrated areas and box joints, both of which are likely to add to the degree of contamination adhering to the instrument, presenting a greater challenge to the cleaning protocol.

In conclusion, this study's findings indicate that the sensitivity level of these tests is relatively poor, with negative findings resulting from tokens where proteinaceous contamination was clearly visible by the application of the EDIC/EF microscopy and the SYPRO Ruby protein stain.¹⁸ Therefore, although they may represent a useful tool for the assessment of general instrument cleanliness, the application of more sensitive detection methodologies should be applied for 'high-risk' devices such as neurosurgical instruments to reduce the risk of iatrogenic infection.

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