

**Department of Health Funded Research on  
Decontamination of Surgical Instruments:**

**Progress Review 2008**

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1	INTRODUCTION .....	4
2	ASSAYS FOR THE DETECTION OF PROTEIN AND PRION CONTAMINATION ON SURFACES .....	6
2.1	Colorimetric test for protein detection .....	6
2.2	Enzyme-linked immunosorbent assay (ELISA) detection of prion protein.....	8
2.3	Fluorometric detection of prion protein .....	11
2.4	Magnetic acoustic resonant sensor technology (MARS) .....	17
3	DETECTION OF INFECTIVITY ATTACHED TO IMPLANTS .....	19
3.1	Development of animal model bioassay .....	19
3.2	Transmission of infectivity through dentistry .....	23
4	ANALYSIS OF PROTEIN LEVELS ON SURGICAL INSTRUMENTS ....	25
4.1	Simulated instrument contamination study .....	25
4.2	Small study on surgical instrument contamination .....	25
4.3	Surgical instrument protein contamination survey -2003 updated results .....	27
4.4	Quality control in CSSD washers and instrument design considerations.....	30
4.5	Evaluation of cleaning methods on protein contamination of dental instruments .....	31
5	DEVELOPMENT OF DECONTAMINATION PROTOCOLS .....	32
5.1	Factors affecting TSE infectivity .....	34
5.2	Autoclaving .....	35
5.3	Efficacy of current NHS procedures for protein removal.....	39
5.4	Removal of protein with detergents .....	42
5.5	Proteolytic degradation of TSE Agents .....	45
5.6	Proteolytic degradation of vCJD (Prion Unit) .....	46
5.7	Development and use of contamination /decontamination assays on surfaces .....	47
5.8	Decontamination of luminal endoscopes .....	51
6	GAS PLASMAS AND OTHER PHYSICO-CHEMICAL TECHNOLOGIES FOR DECONTAMINATION .....	54
6.1	Low pressure radio-frequency gas plasma .....	54
6.2	Cold atmospheric pressure plasmas.....	57
6.3	Ozone sterilisation .....	62

6.4	Electro-elution.....	63
7	DEVELOPMENT OF BARRIERS AND SURFACE COATINGS .....	68
7.1	Barriers for ophthalmic devices .....	68
7.2	Diamond-Like Carbon Coatings.....	72
7.3	Photocatalytic coatings.....	76
8	SUMMARY AND CONCLUSIONS.....	80
9	APPENDIX 1.....	82
10	APPENDIX 2.....	84
11	APPENDIX 3.....	100
12	APPENDIX 4.....	102
13	APPENDIX 5.....	105
14	APPENDIX 6.....	106

# **1 INTRODUCTION**

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases which have given rise to public health concerns in the UK and world-wide. The TSE agents that cause the brain-wasting diseases BSE (mad cow disease), scrapie in sheep and Creutzfeldt-Jakob disease (CJD) in humans are all extremely resistant to inactivation. This means that procedures that would destroy the infectivity of bacteria and viruses appear to be largely ineffective against these agents. The mode of transmission of the 'natural' diseases (i.e. scrapie and sporadic CJD) is unknown.

The origin of the 'first case' of BSE is also unknown, but there is little doubt that the epidemic of BSE in cattle was caused by the addition of infected meat and bone meal as a source of protein in cattle feed. In 1996 it became clear that the strain of agent that causes BSE had transmitted to a small number of humans. The new disease was called variant CJD (vCJD). Again, how it transmitted is not known but many believe it involved the consumption of infected meat products from cows.

There is however another way these diseases can be transmitted, as adverse effects of medical treatment: iatrogenic transfer. This has occurred when tissues from an infected human are transferred to another human patient for medical reasons—for example growth hormone extracts and blood transfusions. It may also occur when surgical instruments that have been contaminated by infected tissues are re-used. Of course, the tissues or instruments in question will have been treated with standard decontamination procedures but these do not effectively destroy the TSE agents.

The extent of transfer of BSE from cows to humans is unknown: the assumption has been made that exposure was quite widespread. Despite continuing efforts, the prevalence of human TSEs in the UK population has not been reliably determined. As a matter of urgent precaution, it has been concluded that measures are needed to prevent potential iatrogenic transfer of vCJD from one individual to another.

In 2001, the Department of Health and the UK TSE Joint Funders identified the need to commission research on: diagnostics to detect and distinguish between all forms of CJD; look at the spread of disease through routes that have a theoretical risk of secondary transmission (e.g. blood, tissue, organ transplants, and surgical instruments); and examine and develop technologies capable of decontaminating surgical instruments with emphasis on protein deactivation and prion removal. A number of projects were commissioned and contractors were formed into the Working Group for Research into the Decontamination of Surgical Instruments (DECON), with Professor Don Jeffries as Chair (Appendix 3). The DECON group have met biannually since 2001. This has ensured productive interchange between contractors and allowed the DH to remain well informed on progress towards goals.

The report *'Department of Health Funded Research on the Decontamination of Surgical Instruments: Current Progress and Future Needs'* summarised the research of the DECON group up to 2005. The research programme focused

on the following areas: detection of protein and prion contamination on surfaces and surgical instruments; assessment of current NHS Sterile Service Department procedures; development of technologies for decontamination; and the development of barriers and novel coatings for medical devices and surgical instruments.

The report also provided pointers to future research priorities in this field such as definition and measurement of appropriate cleanliness, and how that might be achieved by cleaning protocols. By definition, if all matter acquired during interventional procedures are removed from the surface there can be no TSE infectious agent present, so the ideal for the cleanliness of re-useable surgical instruments is to obtain this 'chemically clean' status. There are now procedures that may achieve this, such as the use of gas plasmas.

The outputs from the results of several projects have been published in peer reviewed journals and have provided information for the Engineering and Science Advisory Committee into the Decontamination of Surgical Instruments including Prion Removal (ESAC-Pr). Some of the research findings have also been used by the National Institute for Health and Clinical Excellence (NICE) who published guidance in November 2006 on "Patient safety and reduction of risk of transmission of Creutzfeldt-Jakob disease (CJD) via interventional procedures." After consideration of the report the Spongiform Encephalopathy Advisory Committee (SEAC) issued a position statement on methods to evaluate decontamination technologies for surgical instruments [www.seac.gov.uk/statements/statement310806.htm](http://www.seac.gov.uk/statements/statement310806.htm).

Three years on, the report has been updated to include much of the research presented at the twice yearly DECON meetings (Appendix 4), published papers (Appendix 2), final reports and answers from questionnaires sent to the contractors.<sup>1</sup> The 2008 report will provide information for the Department of Health with the aim of progressing these results from the research field into practical application within an NHS setting.

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<sup>1</sup> The DECON group also covers elements of protein molecular biology relevant to decontamination, for example the work of Dr Igor Bronstein on PrP structure and characterization. An update on this work is provided in Appendix 1.

## **2 ASSAYS FOR THE DETECTION OF PROTEIN AND PRION CONTAMINATION ON SURFACES**

When surgical instruments are taken into a Sterile Services Department (SSD), either after surgery or new, they are cleaned and disinfected in a mechanical washer-disinfector (WD) with the addition of a chemical additive (detergent, rinse aid, disinfectant) and/or an enzymatic cleaner and possibly a ultrasonication step. Once dry, the instruments tend to be inspected visually by trained operators and passed for any residual soiling or mechanical failure before being packaged and sent for sterilisation. However visual inspection does not provide for detection of transparent residues or of residues located in lumens that are not, or only poorly, amenable to inspection. Additional quantitative checks of the cleaning performance must be carried out on a regular basis.

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. The effectiveness of these methods relies on both the effective sampling of the instruments and the sensitivity of the tests employed.<sup>2</sup>

A number of projects have looked at the efficacy of colorimetric test for protein detection and at developing novel methods for protein and prion detection on surgical instruments, to allow validation of cleaning and decontamination procedures. The techniques used and results from these projects are described in the following sections.

### **2.1 Colorimetric test for protein detection**

#### **2.1.1 Ninhydrin test evaluation**

The 'standard' Ninhydrin test used for the detection of residual protein in many SSDs has been evaluated and its analytical performance 'validated' by Professor Perrett at the William Harvey Research Institute at Barts' and London School of Medicine and Dentistry. It was found that the test was insensitive in detecting intact proteins when the blue/purple colour was evaluated visually. In particular, it was found that the test was 100 times less sensitive in detecting protein standards, such as bovine serum albumin (BSA), than it was in detecting the free amino acid, arginine, with which the commercial tests are calibrated.

#### **Key findings**

- Sensitivity of the test is dependent on the type of protein tested.

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<sup>2</sup> Lipscomb IP, Sihota AK, Botham M, Harris KL & Keevil CW. (2006c). Rapid method for the sensitive detection of protein contamination on surgical instruments. *J Hosp Infect*, **62**: 141-148.

- Results suggest that the test is misleading.
- The researchers suggest that DH should recommend that its use be discontinued.

This project has ended.

### **2.1.2 Ninhydrin and Biuret test evaluation**

Professor Keevil's Group at Southampton University have evaluated two commercially available contamination tests, the Biuret test kit (Pro-TECT M, Biotrace, Bridgend, UK) and Ninhydrin kit (Albert Browne Ltd, Leicester, UK) for their sensitivity to ME7 mouse brain homogenate (MBH) on 'pristine' 316L surgical-grade stainless steel surfaces. Controls were visualised by the application of Episcopic Differential Interference Contrast/Epi-Fluorescence microscopy (EDIC/EF) combined with the sensitive fluorescent reagent, SYPRO Ruby. This stain has been shown to aid rapid visualisation and assessment at low levels of protein contamination on medical devices. (The EDIC/EF method is described in section 2.3.1). The Ninhydrin test displayed a minimum level of detection observed by 75% of volunteers (MLD<sub>75</sub>) of 9.25 µg [95% CI 8.6 to 10.0 µg]. The Biuret test provided better sensitivity, with a MLD<sub>75</sub> of 6.7 µg [95% CI 5.4 to 8.2 µg]. However, using the EDIC/EF microscopy method, photomicrographs showed proteinaceous contamination could be visualised to below 0.4 ng. This demonstrates greater than 10,000-fold increased sensitivity than the Ninhydrin and Biuret test.

From these findings, it is clear that approved colorimetric tests for protein contamination are relatively insensitive compared to EDIC/EF and SYPRO Ruby.

This investigation also demonstrates how large amounts (up to 6.5 µg) of proteinaceous brain contamination could remain undetected and the instruments deemed clean using such methods. The researchers recommend that 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.<sup>3</sup>

### **Key findings**

- Biuret test is more sensitive than Ninhydrin test in detecting ME7 MBH.
- EDIC/EF is 10,000-fold more sensitive than both colorimetric tests.
- Instruments deemed clean have low levels of undetected protein contamination.

### **Exploitation**

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<sup>3</sup> Lipscomb IP, Pinchin HE, Collin R, Harris K & Keevil CW. (2006a). 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. *J Hosp Inf*, **64**: 288-292.



- A simple fluorescent test that is more sensitive towards proteins is currently under development.

## ***2.2 Enzyme-linked immunosorbent assay (ELISA) detection of prion protein***

### ***2.2.1 Indirect ELISA***

Dr Denise Dear and colleagues at the University of Cambridge have developed a custom designed enzyme-linked immunosorbent assay (ELISA) to enable the detection of recombinant PrP (rPrP) and the disease-associated prion protein (PrP<sup>Sc</sup>) bound to surgical steel.

The assay has a detection limit of 175 pg/mm<sup>2</sup> for ovine and murine rPrP bound to surgical steel tokens (Ferritic 430BA). Adsorption can be detected both when rPrP is alone, or in the presence of multiple proteins. The assay uses commercially available antibodies and incorporates a unique magnetic transfer step of the steel tokens which act as the adsorption surface. In addition, the assay can be used to detect conformational changes of the adsorbed protein: a time-dependent conformational change was observed during the adsorption of rPrP to surgical steel. This conformational change was species-specific and varied between the alpha-helical and beta-forms of recombinant protein. Adsorption to surgical steel of rPrP containing a higher percentage of beta-sheet was also studied and preliminary examination showed a time-dependent conformational change occurring in the opposite direction to that of the alpha-helical form of rPrP. These factors need to be borne in mind when designing an ELISA for rPrP detection. The content and concentration of the biological material in which rPrP is embedded has a significant influence on the detection ability of the test. This was illustrated when difficulties were encountered in detecting rPrP using a variety of antibodies when it was placed in undiluted brain homogenate. Dilution of brain homogenate was found to be necessary to facilitate detection of rPrP using the ELISA. This result would be expected as undiluted brain homogenate has a high fat content which would lead to masking of key epitopes.<sup>4</sup>

#### ***Key technical features***

- The ELISA is ready for use with a process time of 2 hours.
- The ELISA has a sensitivity of approximately 175 pg/mm<sup>2</sup> for rPrP.
- Prion protein can be detected directly on surfaces.
- Dilution of brain homogenate is necessary for rPrP detection.
- Most laboratories will be familiar with this technique, so it could be assimilated quickly.

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<sup>4</sup> Final report. Grant 1217117. Adsorption of Prion isoforms to Stainless Steel Surfaces: Implication for Surgical Decontamination Procedures 1999-2005.

### ***Future work***

- This technique needs to be adapted to detect PrP<sup>sc</sup> in the presence of an excess of PrP<sup>c</sup> in tissue. This would allow the presence of PrP<sup>sc</sup> to be unhinged from infectivity, especially if the work was performed in conjunction with the originally proposed animal study (see final report).
- Further work would investigate the detection of normal versus disease-associated PrP<sup>c</sup> (glycosylated) from tissue. This requires immunoprecipitation of PrP<sup>c</sup> and PrP<sup>sc</sup> to allow their concentration using appropriate antibodies or ligands (available from the IOB and Veterinary School in Cambridge).

This project has ended.

### ***2.2.2 ELISA with adenylate kinase***

A group led by Dr Neil Raven at the Health Protection Agency (Porton Down) have developed a high sensitivity ELISA, based on thermostable adenylate kinase (tAK) enzymes derived from genetically engineered bacteria, for the detection of prion material on surgical instruments.

#### ***2.2.2.1 Development of a high sensitivity ELISA for rPrP***

The ELISA format is based on the bioluminescent detection of adenosine triphosphate (ATP) generation by the enzyme adenylate kinase (tAK). Studies have shown that in complex biological mixtures, the heat inactivation of endogenous AK activity reduced background levels effectively to zero. As such, sensitive detection of rPrP alone and in the presence of neuronal tissue, blood, sera and on the surface of surgical grade stainless steel was demonstrated.

#### ***Key technical features***

- In a fully optimised tAK ELISA for rPrP, a detection limit of approximately 1.5 pg has been observed.
- The ELISA system has a sensitivity of 0.5-1 pmoles of rPrP in the assay solution.
- In non-infectious studies, the anti-prion antibody – tAK conjugates have been used to detect spiked rPrP down to 0.5 ng in the presence of 0.05 mg of homogenised mouse brain tissue.
- On surgical steel discs the assay has been able to detect rPrP to below 10 ng.
- The ELISA can detect protein directly on the surface of instruments.

#### ***2.2.2.2 Development of a high sensitivity ELISA for PrP<sup>sc</sup>***

A follow on study aimed to repeat the rPrP experiments to determine a detection limit for PrP<sup>sc</sup> in a standard ELISA method and on the surface of

surgical steel. A model with PrP<sup>sc</sup> in BSE-301V infected mouse brain homogenate (iMBH) using the 6H4-AK conjugate was used. The project achieved reasonable levels of detection for infectious brain material although it did not achieve the aim of being able to use the methodology for comparison of standard cleaning processes, due to difficulties in obtaining consistent results on surgical steel.

The detection limit for iMBH was calculated as 320 ng/ml. This would equate to approximately 16 infectious units assuming all of the detected PrP was PrP<sup>sc</sup>. Experiments with iMBH consistently showed a 10-fold higher signal for PrP detection to normal MBH at the same protein concentration, which suggests that around 90% of the signal was due to the disease associated protease-resistant form.

These results were obtained on standard ELISA plates and the assay did not perform as effectively on stainless steel surfaces. A number of problems were encountered with improving the sensitivity beyond the 40 µg/ml value obtained here. The tAK enzymes bind to stainless steel surfaces in their own right, giving rise to relatively high background values. Related immunoassay projects have subsequently made some progress on reducing the background binding to surfaces, by using different tAKs but this was after the completion of this project.

Most assays designed for the detection of PrP<sup>sc</sup> are hampered by the lack of specific antibodies for this form of the protein. Many methods still employ proteinase K as a means of discriminating between the cellular and disease-associated form. There is some evidence that the PrP<sup>sc</sup> in blood and a significant proportion of infectious material in other tissue types, may not be protease resistant. The ELISA study used proteinase K to try to reduce background in both ELISA plates and on stainless steel but without success. Again, this may reflect the inability of proteinase K to access PrP in all forms once bound to surfaces.

The project also briefly explored the suitability of the assay for detection of prion material in blood. The initial studies suggest that the sensitivity of the assay approaches that required for the detection of prions in blood. The current best estimates used by the National Blood Service and others, is that there are around 1-100 infectious units of vCJD prion agent per ml of blood (equating to around 0.1-10 pg/ml prion protein) based on animal TSE models. In order for this method to be useful for diagnosis it would need to address issues of specificity for and/or enrichment of disease-associated PrP.

The project was unable to develop the ELISA to a point where it could be used for comparative testing of cleaning and decontamination processes, largely due to the difficulties of generating consistent results on stainless steel. However, the project did assist in identifying that tAKs in their own right might be useful for evaluating protein removal processes.<sup>5</sup> Subsequent projects have used the surface binding properties of the tAKs to develop rapid, quantifiable measures of protein removal during cleaning and

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<sup>5</sup> Final report. Grant 1217034. Validation of TSE Contamination 2004-5.

decontamination. This work is the subject of advanced commercial discussions.

### ***Key technical features***

- The detection limit for iMBH was calculated as 320 ng/ml on ELISA plates.
- The detection limit for iMBH is 40 µg/ml on stainless steel discs.
- tAK enzymes bind to stainless steel resulting in high background values.
- The surface binding properties of tAK form the basis of rapid methods for quantifying cleaning efficacy. These may be relevant for assessing relative removal of proteins in general, and prions specifically, by different chemistries and types of process.
- Evidence that the PrP<sup>Sc</sup> in blood and a proportion of infectious material in other tissue types may not be protease resistant.

This project has ended.

## ***2.3 Fluorometric detection of prion protein***

### ***2.3.1 Episcopic Differential Contrast Microscopy***

Professor Keevil's group at the University of Southampton have developed a novel microscopy principle, Episcopic Differential Interference Contrast microscopy coupled with Epi-Fluorescence (EDIC/EF), that provides unique, sensitive, and high quality visualisation of opaque and often highly convoluted surfaces without the requirement of oil immersion or a coverslip. The group have carried out studies using this technique in combination with sensitive fluorescent dyes such as the ruthenium-based stain SYPRO Ruby. This reacts non-covalently with proteins. The thiazole derivatives thioflavin T and thioflavin S, have also been used as stains, both of these have been advocated for the detection of amyloid and beta-pleated sheet proteins, indicative of PrP<sup>Sc</sup>, in both histopathology and biochemical assays.

#### ***2.3.1.1 Detection of total protein contamination by EDIC***

##### ***SYPRO Ruby stain of brain tissue***

Studies with 316L stainless steel coupons have shown that diluted brain homogenate can be rapidly visualised *in situ* by EDIC/EF when used in combination with SYPRO Ruby. Eight independent observers looking at 12 replicate samples were able to visualise a protein threshold value of 400 pg/mm<sup>2</sup>. 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 was 175 pg/mm<sup>2</sup> [95% CI 104–286 pg/mm<sup>2</sup>] at MLD<sub>75</sub>

(detectable by 75% of observers), and of 85 pg/mm<sup>2</sup> [95% CI 67–112 pg/mm<sup>2</sup>] for MLD<sub>50</sub>.<sup>6</sup>

### **2.3.1.2 Detection of PrP<sup>Sc</sup> in brain tissue and spleen on surgical steel by Thioflavin T/S stain**

The thiazole derivatives thioflavin T and thioflavin S have been reported to display an affinity to bind with aggregates of prion protein. The effectiveness of thioflavin T and thioflavin S as stains for brain homogenate and spleen tissue from ME7 (murine scrapie) infected mice was examined in a series of experiments. Tissue samples were smeared or spotted onto stainless steel tokens and ‘cleaned’ Spencer Wells Forceps.<sup>7</sup>

Brain tissue sections (10 µm) were placed onto stainless steel tokens and stained with the thiazoles. Large plaques of PrP<sup>Sc</sup> and ‘mini-plaques’ with a diameter of less than 1µm were readily visible using EDIC/EF microscopy 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. (The immunocytochemical characteristics of monoclonal antibodies from Dr Jacques Grassi’s group (Commissariat à l’Energie Atomique, Saclay, France), raised against scrapie-associated fibrils had been previously investigated). Thioflavin T displayed a superior signal to noise ratio compared with thioflavin S when bound to amyloid, and was used in all the subsequent experiments.

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. No thiazole staining was observed in normal brain homogenate.

By applying the known biological characteristics and physical dimensions of the mouse brain, the group were able to estimate that the thioflavin T-positive, 1 µm aggregates, which are clearly visible, are theoretically composed of less than 100 fg protein, which equates to around 2 attomoles PrP<sup>Sc</sup>.

Immunocytochemistry by Western blotting was conducted in parallel with the EDIC/EF technique and showed that the microscopy method was at least 2 log (i.e. 100-fold) more sensitive than Western blotting.<sup>7</sup>

The thiazole dyes do not discriminate between amyloid plaques composed of PrP<sup>Sc</sup> and plaques that might be composed of other amyloidogenic fibrils such as Aβ, a major component of Alzheimer’s disease pathology.

### **2.3.1.3 Thioflavine T/ SYPRO Ruby dual staining**

The group have developed a dual staining procedure, Thioflavine T/ SYPRO Ruby used in combination with the (EDIC/EF) microscope to increase the resolution of the amyloid material in tissues and on contaminated surfaces,

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<sup>6</sup> Lipscomb IP, Sihota AK, Botham M, Harris KL & Keevil CW. (2006c). Rapid method for the sensitive detection of protein contamination on surgical instruments. *J Hosp Infect*, **62**: 141-148.

<sup>7</sup> Lipscomb IP, Hervé R, Harris K, Pinchin H, Collin R & Keevil CW. (2007a). Amyloid-specific fluorophores for the rapid, sensitive *in situ* detection of prion contamination on surgical instruments. *J Gen Virol*, **88**: 2619-2626.

and discriminate between PrP<sup>Sc</sup> and other proteins for rapid scanning of instrument surfaces. Double-blind studies using various dilutions of ME7 infected brain in normal brain homogenate produced a dose-related signal on contaminated surgical stainless steel surfaces, as assessed by image analysis. The sensitivity of the method proved over 2 log (100-fold) better compared to the classic Western blot procedure with the same prepared samples. It is believed that the sensitivity improvement is actually greater since testing instrument surfaces by Western blot relies on swabbing the contaminated surface, and it was shown in previous studies that PrP<sup>Sc</sup> binds to surfaces more strongly than other proteins. Moreover, if the instrument is serrated or pitted then it is more difficult to elute biological deposits from the surface for analysis. The detection limit for the new procedure was demonstrated to be <100 fg PrP<sup>Sc</sup>; which is the equivalent of 2 attomoles. Therefore, this new sensitive microscopy procedure is rapid and can be applied directly to instrument surfaces to check for amyloid contamination.

Thiazole derivatives—which have been shown to bind to  $\beta$ -pleated sheets present in prion protein fibrils—have been applied both to brain and spleen sections, on both glass and surgical steel tokens, and have proven to provide a more sensitive detection method than immunocytochemistry. Calculations of sensitivity indicate that sub-micron plaques (below 1 pg of prion protein, which equates to around 200 infectious units) can be detected.

### ***Key technical features***

- Whole instruments can be examined quickly, easily and in 3D.
- Curved and/or serrated surfaces can be scanned.
- Very rapid assessment by trained operators.
- Images can be captured electronically.
- Detection of contamination carried out *in situ* without need of swabbing or elution of contaminant.
- SYPRO Ruby shown to have far better sensitivity than other colorimetric stains.
- SYPRO Ruby cannot discriminate whether prion amyloid is present. Good stain for total protein and 10000-fold more sensitive than Ninhydrin.
- SYPRO Ruby detection level for total protein of <400 pg/mm<sup>2</sup>.
- Thioflavine T can detect prion amyloid plaques. Detection level 10 pg protein/mm<sup>2</sup> of test surface.
- Dual staining with Thioflavine T/ SYPRO Ruby has detection of <100 fg PrP<sup>Sc</sup>; approx. 2 attomoles.
- The stain itself is easy to apply and staining procedure takes 30 minutes.
- Stains can be removed with detergents or conventional solvents.

- Stains have no known toxicity at the levels used, but a formal toxicity assessment may need to be carried out.

### ***Exploitation of research and commercialisation***

Southampton University and Best Scientific have filed a patent on the sensitive microscopy procedures and design of the equipment, with the approval of the Department of Health. Microgen Bioproducts as a partner in the project have been granted the licence to market this technology.

### ***2.3.2 Fluorometric detection with fluorescein based fluorophores***

Research on the development of fluorometric detection methods for prion protein is being conducted by the MIDAS group at the Universities of Edinburgh and Heriot Watt. The work can be separated into three areas: methods for efficient fluorescent labelling of proteins on surfaces; development of sensitive spectrofluorometric methods of detection; and application of these methods to the design of a rapid surface scanning spectrophotometer.

#### ***2.3.2.1 Fluorometric detector***

Novel fluorescent probes have been developed which will bind covalently to surface proteinaceous material. Initial studies were conducted on commercially available fluorescent dyes, fluorescein and Coumarin 6. These studies were then extended to fluorescently labelled bovine serum albumin (BSA) protein on surgical steel. Excitation of the sample and detection of the emitted fluorescence were carried out using a photon counting fluorescence spectrometer (Jobin-Yvon-Horiba Fluoromax-P). Fused silica optical fibre bundles, terminated in collimating lenses, were used to convey the excitation light from the spectrometer to the sample, and the fluorescence from the sample to the spectrometer. The sample was mounted on a linear motorised translation stage.

#### ***2.3.2.2 Detection of fluorescein on metal surfaces***

Using fluorescein as the derivatising agent, it was shown that for a scanning rate of 12 cm/min,  $5 \times 10^8$  fluorophore molecules in a 2 mm diameter spot gave a fluorescence signal of 900 photons/s and a signal-to-noise ratio of 20:1 for fluorescein on an aluminium surface. This gives a conservative estimate of the detection limit as 200 attamoles of fluorophore on aluminium.

In 2004, the detection limit on surgical stainless steel for fluorescein and Coumarin-based fluorophores for a detection area of  $3\text{mm}^2$  was below 100 attamoles/ $\text{mm}^2$  for a signal collection time of 1 second.<sup>8</sup>

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<sup>8</sup> Richardson P, Jones AC, Baxter RL, Baxter HC, Whittaker AG & Campbell GA. (2004). Fluorescence detection of protein residues on surgical instruments; Proc SPIE, **5502**: 291-294.

### **2.3.2.3 Detection of fluorescein labelled BSA**

As a model system for the detection of protein contamination, BSA, fluorescently labelled with fluorescamine, was deposited and dried onto discs of surgical quality steel. Extrapolation of the signal-noise ratio of 5:1, and a signal integration time of 1 second, gives an estimated detection limit for BSA of 60 fmole/mm<sup>2</sup>.

The detection procedure has been refined with the use of cut-off filters and collimators to reduce the level of Rayleigh scattered or reflected excitation light from the metal surfaces and further improvements are expected using time-resolved fluorescence. In addition, new filtering strategies are being explored to eliminate a small but significant background signal due to Raman scattering generated in the optical fibre bundle and reflected by the metals surface into the emission fibre. At very low sample level, this is the main limiting factor for detection sensitivity.<sup>8</sup>

### **2.3.2.4 Development of fluorescent labels**

#### ***1st generation of fluorescein based reagents***

Synthesis of the first-generation of iodo-activated reagents based on fluorescein has been completed. Work has shown that while, as anticipated, these give virtually no background signal and are highly fluorescent after reaction with amino compounds, they suffer from hydrolytic instability and are insufficiently soluble in water. Second-generation compounds have been synthesised which should address these limitations.

#### ***2<sup>nd</sup> generation of fluorescein based reagents***

Several 'latent' fluorescent reagents based on the fluorescein chromophore have been generated. Two particularly promising compounds increase their fluorescence by ca 100- and 1000-fold on reaction with bound protein, and both of these can be used to derivatise proteins absorbed on stainless steel surfaces. Labelling efficiencies are about 30% for typical globular proteins adsorbed on surfaces. (These were measured by comparing the fluorescence of proteins labelled on metal surfaces with those of pre-labelled proteins adsorbed on the same surface). The preparation and use of these compounds is the subject of a patent application.<sup>9</sup>

### **2.3.2.5 On-line scanning instrumentation**

In parallel, a 2D scanning spectrofluorimeter for direct quantification of fluorescently labelled proteins on steel surfaces has been designed and constructed. A flatbed design was adopted to avoid the reflectance problems inherent in making fluorescent measurements on polished surfaces, and for ease of use. The novelty of the optical design is the use of collinear optics using a dichroic mirror to filter out the excitation wavelength and thus reduce interference from scattered light. The current version of the instrument (built by the optoelectronics group at Heriot Watt University) which has been in

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<sup>9</sup> MIDAS Final report. Project 0070077. Removal of infective protein residues from medical instruments.



routine use for the past year, has a sensitivity of *ca* 100 attamoles/mm<sup>2</sup> ( $6 \times 10^{-7}$  molecules - about 4 pg) of *in situ*-labelled protein. In comparison – the Ninhydrin swab test, the only currently accepted method in SSD practice for estimating protein load on surfaces, has a sensitivity of 5-10 µg.

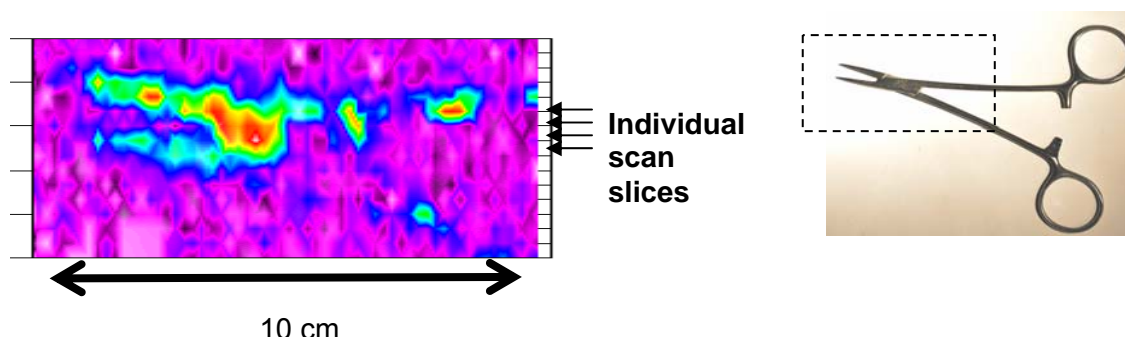


Fig.1. Scan of a pair of 'clean' reprocessed surgical clamps (after labelling with fluorescent compound) individual scan slices take *ca* 1 s. (*Unpublished data*).

### **Key technical features**

- Two fluorescent probes identified for further optimisation.
- Labelling efficiency 30% for globular proteins on adsorbed surface.
- Easy to use, 2D scanner.
- Scanner sensitivity *ca* 100 attamoles/mm<sup>2</sup> ( $6 \times 10^{-7}$  molecules - about 4 pg) of *in situ* labelled protein.

### **2.3.3 Derivatisation of simple amines with o-phthaldialdehyde**

Studies by Professor Perrett and Professor Jeffries at the Barts' and London School of Medicine and Dentistry aim to develop simple but sensitive assays for residual proteins on surgical instruments.

#### **2.3.3.1 Assays for protein detection**

A number of sensitive fluorescent protein assays have been evaluated for the quantification of proteins in solution. Quantitative assays based on NanoOrange and CBCQA (both from Molecular Probes) were compared to an assay already used in Professor Perrett's laboratory for High Performance Liquid Chromatography (HPLC) derivatisation of simple amines, based on o-phthaldialdehyde (OPA) in the presence of a thiol. The first two assays, whilst sensitive, were unstable and gave non-linear responses at the highest sensitivity. OPA/thiol proved equally sensitive. This assay demonstrated a low background fluorescence, was much cheaper, had better excitation and emission wavelengths, and was therefore selected for both optimisation and validation. In its final version, the thiol chosen was N-acetyl cysteine (NAC), and a non-ionic detergent was incorporated into the reagent to increase linearity towards a variety of proteins.

The validation data on the final OPA/NAC assay for proteins demonstrated that the limits of detection of the assay for BSA and globulin were below 50 ng/ml and 9 ng/ml, respectively. The linearity range for a high sensitivity assay of BSA was in the range 0-2.5 µg/ml and the intra-assay reproducibility for BSA at a concentration of 1 µg/ml was of 1.8% relative standard deviation (n=10). The assay requires 600 µl of protein solution and therefore levels of <30 ng of protein (expressed as BSA) can be determined.

#### ***Key technical features***

- OPA/NAC assay can detect most protein species to levels <50 ng/ml.
- All reagents are currently available and cheap, and the technology is well tried.
- The excitation wavelength is easily obtained from a mercury lamp.

## ***2.4 Magnetic acoustic resonant sensor technology (MARS)***

Professor Chris Lowe at the Institute of Biotechnology in Cambridge leads a group who have conducted studies on the adsorption of prion isoforms to stainless steel, plastic, and glass surfaces.

### ***2.4.1 Detection of prions and other proteins by MARS***

Dr. Raj Sethi has developed the magnetic acoustic resonant sensor (MARS) to study the adsorption both of the model protein human immunoglobulin G (IgG) obtained from human serum and of rPrP to surgical stainless steel. By working at the first harmonic frequency of steel at 6.3 MHz, rPrP could be detected at a level of 1.6 ng/mm<sup>2</sup> using MARS. The binding was further confirmed by X-ray photoelectron spectroscopy analyses (XPS), and an ELISA.

Further sensing enhancement (8-fold increase) obtained by working with a simulated surgical steel surface, a chromium coated silica glass device, gave a detection limit of 0.2 ng/mm<sup>2</sup> at the 15th harmonic of 50 MHz. Significant data on a model protein h-IgG and rPrP relating to adsorption/desorption kinetics, surface coverage and conformation changes under a variety of experimental conditions was collected which subsequently led to the evaluation of various cleaning/sterilising protocols for contaminated surgical instruments.

Improved signal processing by making use of a coaxial line resonance in the connection electronics in conjunction with a new high frequency signal generator has enabled the operating frequency to be increased to 600 MHz, to obtain the first acoustic frequency spectrum of rPrP, and of mixed rPrP and

human IgG. The increased sensitivity of detection is estimated to reach 16 pg/mm<sup>2</sup>.<sup>10</sup>

***Key technical features***

- Technology has the ability to yield a protein-specific acoustic fingerprint for proteins adsorbed to surfaces.
- The MARS system has an estimated sensitivity of 10-20 pg/mm<sup>2</sup> for rPrP.
- No staining is required.
- A commercial instrument could be developed which could be hand-held or set into a production line.
- Such an instrument could detect prions directly on surfaces such as simulated stainless steel, quartz and plastic-coated quartz.
- After development commercial units could cost as little as a mobile phone. (The cost at present of a multipurpose commercial laboratory system is approximately £75000).

This project has ended.

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<sup>10</sup> Final report. Project 217117. Adsorption of Prion isoforms to Stainless Steel Surfaces: Implication for Surgical Decontamination Procedures 1999-2005.

### **3 DETECTION OF INFECTIVITY ATTACHED TO IMPLANTS**

There is clear evidence of cases of iatrogenic Creutzfeldt–Jakob disease (CJD) arising from surgery where neurosurgical-instrument contamination has been implicated. By using animal models, it has been shown that transmission of infectivity can occur via stainless-steel surfaces. At the Moredun Institute, work carried out by Dr. Lynne Bountiff, Louise Gibbard and Dr. Hugh Reid aimed to establish an animal model of iatrogenic transfer of a TSE by a peripheral route, and thereby create a procedure to evaluate the efficacy of protocols used for the decontamination of instruments.

#### **3.1 Development of animal model bioassay**

A small animal model using “infection coated” stainless steel implants was used to measure TSE infectivity by bioassay rather than relying on the detection of PrP<sup>Sc</sup> alone. Unlike other published wire or implant bioassays, this procedure allowed quantification of attached infected tissue using dry weight measurements.

Under current guidelines, validation for decontamination procedures requires a clearing of 3 logs of infectivity. Since this procedure provides a measure of dose of agent per unit wet weight of tissue before decontamination, it is possible to set up a validatable assay of decontamination. Implants were treated to mimic “inadequately” decontaminated stainless steel surgical instruments.

##### **3.1.1 Measurement of infectivity attached to implants**

###### ***Experimental protocol***

A ten-fold dilution titration was set up to determine the sensitivity range of the bioassay in hamsters and mice. Wet inocula consisting of hamster or mouse brains (20% weight/volume) homogenised in 0.32 M sucrose at dilutions of  $10^{-2}$  to  $10^{-6}$  were used. For preparing controls for future supplies of inocula, and to provide a comparison with published data, samples using a  $10^{-2}$  dilution of homogenate in saline were injected intracerebrally. The inoculum was applied to an implant (2mm diameter 316L stainless steel spheres) by immersing the implant in a standardised volume of inoculum, and drying at room temperature (varying between 20°C and 24°C) to constant weight.

The implant bioassay proved to be very successful: residual infected tissue, attached to a surgical stainless steel implant, can reliably transmit 263K scrapie to hamsters and 79A scrapie to C57bl mice by peripheral routes. The hamster model was chosen for further work as the clinical end-point was shorter, therefore the experiments could be performed quickly.

The dry weight of the infection-coated spheres indicated that the equivalent of between 0 and 5 µl of wet inoculum had adhered. Spheres with loading in the range of 1 to 3 µl were implanted. In these experiments, three out of ten intraperitoneal (i.p.) implants in hamsters were found to have migrated post

implantation to a ventral abdominal subcutaneous (s.c.) position. However, in all the mice and in the sensitivity of implant bioassay experiment, all but one implants intended for i.p. were located i.p. post mortem.

### **3.1.2 Factors affecting efficiency of transfer of infection**

The animals were monitored for clinical signs of disease throughout the course of the experiment. Scoring was based upon well developed and documented systems.<sup>11,12</sup> The clinical signs and spongiform lesions in the hamsters broadly conform to published evidence and confirmed the infection with scrapie. One of the other well documented characteristics of scrapie is the hierarchy of efficiencies of infection by different routes. For hamsters the published trend is: i.c.>i.p.>s.c. The incubation periods for  $10^{-2}$  wet inoculum of both mouse and hamster models by the i.c. route were in agreement with published data, for hamsters this was at 65 days post injection. The incubation periods in hamsters for all the implants with dried on homogenate were much shorter than equivalent wet inoculum, i.e. between 86 and 105 days against at least 130 days. Thus, it is apparent that there is a greatly increased efficiency of infection for the peripheral route observed with the 20% dried on homogenate. These observations have now been repeated over a series of three separate experiments. There is also evidence that the temperature at which implants are dried may have some small influence over the incubation period. The results indicate that although the assay is easy to perform, the implant bioassay must be carried out under strictly controlled conditions.

### **3.1.3 Sensitivity of the hamster implant bioassay**

In the hamster tenfold dilution titration, all the animals in the  $10^{-2}$  group and one animal from the  $10^{-4}$  dilution reached clinical endpoint, thus the titre is  $10^{2.75}$  infectious unit (i.u.) per sphere or  $10^4$  i.u. per 50 $\mu$ l. However, as clinical disease did not extend as far down the dilution series as was expected, a dose response curve could not be constructed.

A second titration was therefore set up. The effective range of the assay using a four fold dilution of infectivity, and inserting 4 spheres/animal, was from a 1:4 to 1:256 dilution. In the first titration, the spheres were coated with an average of 1.1 mg (infected hamster brain homogenate (HBH))/sphere. In a collaboration with Professor Keevil's group, experiments were conducted using SYPRO Ruby stain and EDIC to determine the extent of homogenate covering the sphere. A coverage of between 80% and 90% for the spheres in the first titration was established. In this second titration the coating range was between 1 and 5 mg HBH per four spheres with the overall average at 1.88 mg HBH per four spheres. The diluent was 20% normal hamster brain in 0.32M sucrose. Using the Karber formula this gave a final titration of  $10^{1.9}$  i.u. per four spheres. While this is quite consistent with the first titration in terms of the dose administered to each hamster, it also indicates that in this

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<sup>11</sup> Fraser H & Dickinson AG. (1968). The sequential development of the brain lesion of scrapie in three strains of mice. *J Comp Pathol*, **78**: 301-311.

<sup>12</sup> Marsh RF & Kimberlin RH. (1975). Comparison of scrapie and transmissible mink encephalopathy in hamsters. II. Clinical signs, pathology and pathogenesis. *J Inf Dis*, **131**: 104-110.

preparation the coverage by weight was approximately 25% per sphere of that achieved in the first titration.

In summary, of significance both to scrapie pathogenesis and to hospital decontamination protocols, dried on infected tissue has a much greater efficiency of infection (> 5,000 times) than wet inoculum by both i.p. and s.c. routes of infection. Interestingly this only applies to infected material present in high concentrations (20% dried on homogenate). At higher dilutions (1 in 100 dried on homogenate) the dynamics of infection and replication change so that the increase of efficiency is around 15 times.

### ***Key findings***

- A validatable implant bioassay for iatrogenic transfer of TSE by peripheral routes has been established.
- The i.p. implant has the advantage over the i.c. wire assays in that the infectious dose on the implant can be measured.
- Incubation period cannot be used to calculate titre with dried on infectivity unless a titration of dried on infectivity is also made.
- Dried on infected tissue has a much greater efficiency of infection (> 5,000 times) than wet inoculum by both i.p. and s.c. routes of infection.
- Efficiency of infection is influenced by concentration of infectious material in the sample.
- The change in the titre to incubation period dynamics as demonstrated by the increased efficiencies of infection in all the implant bioassays so far reported needs careful consideration when interpreting all decontamination of surgical instrument bioassays.

### ***3.1.4 Decontamination protocols tested***

The results indicate that extrapolation from a titration curve for infectivity treated in one way, is unsafe for risk assessments of the efficacy of decontamination of infection by a different treatment. Titration of each procedure will therefore be required.

Notwithstanding this, five decontamination protocols were tested in the hamster model with 263K scrapie in 20% hamster brain homogenate:

1. Intraperitoneal implant coated with HBH, as positive control.
2. Intraperitoneal implant coated with HBH, allowed to dry then washed with detergent.
3. Intraperitoneal implant coated with HBH, allowed to dry then autoclaved and washed with detergent.
4. Intraperitoneal implant coated with HBH, allowed to dry then washed using novel gentle chemical treatments.
5. Intraperitoneal implant coated with HBH, allowed to dry then exposed to plasma ablation cleaning system.

The group 4 treatment is useful for delicate pieces of equipment. The group 5 study is a collaboration with Dr. Helen Baxter at Edinburgh University (see 3.1.5). This plasma ablation procedure has already been shown to be very effective at cleaning metal.<sup>13</sup>

The animals were monitored for clinical signs of disease and were culled because of terminal neurological disease or if unaffected at the end of the experiments. Confirmatory histological examination of the central nervous tissue was conducted at termination.

### **3.1.5 Effects of cleaning treatments on infectivity**

The incubation period of the disease in hamsters in the different experimental regimes is shown in Table 1.

**Table 1. *In vivo* analysis of infectivity of 263K contaminated stainless steel spheres before and after decontamination treatments**

Treatments of contaminated spheres	No of terminal hamsters /total number	Incubation period +/- SD days
Group 1 (Control)	5/5	92 +/- 3
Group 2 (Detergent)	0/5	>466*
Group 3 (Autoclave then detergent)	5/5	202 +/- 28
Group 4 (Novel gentle chemical)	0/5	>466 *
Group 5 (RF-gas plasma)	0/5	>466 *

SD = standard deviation

\*All animals in these groups were still clinically sound at the end of the experiment

These results demonstrate that TSE infectivity can be transmitted via surgical steel implants. All the hamsters in the control group succumbed to the disease by 92±3 days. Treating the spheres with detergent alone, or chemical treatment or RF gas plasma was effective at removing TSE infectivity. However autoclaving the spheres before detergent washing was much less effective at removing infectivity, this may be due to autoclaving affecting the TSE strain, for example by increasing its resistance to heat inactivation or to the detergent wash.

### **Key technical features**

- The bioassay can be used to assess decontamination protocols.
- Appears to be very sensitive.
- Detergent, chemical treatment and RF gas plasma effective at removing infectivity.

<sup>13</sup> Whittaker AG, Graham EM, Baxter RL, Jones AC, Richardson PR, Meek G, Campbell GA, Aitken A & Baxter HC. (2004). Plasma cleaning of dental instruments. *J Hosp Infect*, **56** (1): 37-41.

- Autoclaving affects properties of TSE strain and in combination with a detergent wash is less effective at removing infectivity.

#### ***Future work***

- Further studies should compare the efficiency of dried and wet materials for their ability to transmit infectivity. The dynamics of infection with wet and dry inocula are proving to be unpredictable.
- The assessment of further decontamination protocols should be decided.

#### ***3.1.6 Effect of RF gas plasma on infectivity on surgical instruments and spheres***

A collaborative study with the Edinburgh University group, lead by Dr Helen Baxter investigated the use of radio-frequency (RF) gas plasma as a method of removing both the protein debris and TSE infectivity. Stainless-steel spheres contaminated with the 263K strain of scrapie and a variety of used surgical instruments, which had been cleaned by a hospital SSD, were examined both before and after gas plasma treatment, using SEM and energy-dispersive X-ray spectroscopic analysis. Transmission of scrapie from the contaminated spheres was examined in hamsters by the peripheral route of infection. RF gas plasma treatment effectively removed residual organic residues on reprocessed surgical instruments and gross contamination both from orthopaedic blades and from the experimentally contaminated spheres. *In vivo* testing showed that RF gas-plasma treatment of scrapie-infected spheres eliminated transmission of infectivity. These results demonstrate that 'stubborn' residual contamination on surgical instruments and infectivity of the TSE agent adsorbed on metal spheres could be removed effectively by gas plasma treatment.<sup>14</sup>

### ***3.2 Transmission of infectivity through dentistry***

The HPA, Glasgow Dental School and Leeds Dental Institute have looked at the distribution of infectivity in the oral tissue of mice as a means of assessing the potential risk of vCJD transmission through dentistry.

The study used a clinically relevant model of BSE-301V and VM indicator mice of known susceptibility. Two routes of challenge were investigated: duodenal and gingival. In the duodenal study, mice were challenged with infected mouse brain homogenate (iMBH) inoculated directly into the lumen of the small intestine in the region of the duodenum, to ensure no direct contamination of the oral tissues. The mice were killed at intervals over a 24 week time-course and a range of clinically relevant oral tissues (including

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<sup>14</sup> Baxter HC, Campbell GA, Whittaker AG, Jones AC, Aitken A, Simpson AH, Casey M, Bountiff L, Gibbard L & Baxter RL. (2005). Elimination of transmissible spongiform encephalopathy infectivity and decontamination of surgical instruments by using radio-frequency gas-plasma treatment. *J Gen Virol*, **86**: 2393–2399.



dental pulp and gingival margin) were removed, processed and re-inoculated into indicator mice to observe for infectivity. The primary challenge proved to be a very efficient route of infection with a 100% attack-rate and a mean incubation to clinical disease of  $157 \pm 17$  days (c.f. 120 days for the equivalent titre inoculated intracranially). Infectivity was observed in all oral and control tissues at a range of titres during both pre-clinical and clinical stages of the disease.

In a second study, VM mice were inoculated using endodontic files coated with 10% BSE 301V MBH for 30 minutes. The homogenate had been air dried for one hour, then inserted into the VM mouse gum-line for 5 minutes and then removed. The primary challenge via the gum was very efficient with an attack rate of 100% in the first group and over 90% in the second group and a terminal mean incubation to clinical disease of  $234 \pm 33.7$  days.

This study demonstrates the spread of prion infectivity from both the small intestine and the gingiva to the oral cavity in the mouse. Further results are awaited. However, results so far support the ongoing requirement for risk assessments looking at the potential for vCJD transmission via dental procedures and associated studies looking at the effectiveness of decontamination and re-use of dental instruments.

## 4 ANALYSIS OF PROTEIN LEVELS ON SURGICAL INSTRUMENTS

Several groups have examined surgical instruments which have been in circulation for levels of total protein contamination to assess the effectiveness of routine cleaning and disinfection in hospitals. Others studies have tried to simulate protein contamination on instruments.

### 4.1 Simulated instrument contamination study

Studies by Professor Perrett and Professor Jeffries were designed to estimate the initial level of tissue and protein contamination on surgical instruments. In order to evaluate the level of protein contamination that can occur on simple instruments, kidney tissues or animal brains were manipulated with standard stainless steel instruments and the mass of tissue deposited (both wet and dry weight) was determined. From the non-normal distribution of the data presented in Table 2, it is clear that the level of contamination is very variable. On forceps and tweezers up to 14 mg (d.w.) of brain tissue can be deposited on the instruments. Similar studies were also performed on twenty stainless steel dental files of various gauges. The mass of total protein per instrument was 250 µg (range 50-500 µg).

Table 2. Mass of tissue deposited on various stainless steel instruments

Instrument type	Mass of Tissue deposited (mg)			
	Kidney		Brain	
	Wet weight	Dry weight	Wet weight	Dry weight
Surgical Blades	5.7 ± 2.4	2.0 ± 0.8	nd	nd
Forceps	60.4 ± 45.5	8.6 ± 13.4	33.4 ± 32	14.8 ± 15
Tweezers	11.7 ± 6.8	1.9 ± 0.9	43.0 ± 15	14.3 ± 5

nd: not determined

### 4.2 Small study on surgical instrument contamination

A double blind study of surgical instruments has also been undertaken by Professor Keevil's group at the University of Southampton. 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 (WD) cleaning procedures and had been deemed to be clean.<sup>15</sup>

#### Scoring criteria

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<sup>15</sup> Lipscomb IP, Hervé R, Harris K, Pinchin H, Collin R & Keevil CW. (2007a). Amyloid-specific fluorophores for the rapid, sensitive *in situ* detection of prion contamination on surgical instruments. *J Gen Virol*, **88**: 2619-2626.

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 then examined using the EDIC/EF microscope at multiple points over their surface. A contamination index was then established describing contaminant particle size, field of view coverage and protein/mm<sup>2</sup>. This index enabled rapid assessment of the degree of contamination apparent on each region of interest. Class 0 describes no detectable protein contamination while class 4 can exceed 21 mg protein/mm<sup>2</sup> covering over 50% of the observed instrument surface.

**Table 3. The defined parameters and equivalent protein concentrations for the contamination index**

Contamination index	Particulate height (µm)	Particulate width (µm)	FOV Coverage (%)	Protein/mm <sup>2</sup>
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	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<sup>2</sup>); PE, protein equivalent soil that did not stain with SYPRO Ruby. Protein/mm<sup>2</sup> was calculated using the information that a 1mm-diameter area of protein with an average molecular weight of 30 kDa and 3 mm in height has been calculated to be approximately equivalent to 1 pg (data not shown).

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.

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 1-2). The overall mean contamination index value for all the instruments was 2.8. Overall, the EDIC/EF procedure was much more sensitive than visual observation.

### **Key findings**

- This study demonstrates the ineffectiveness of visual inspection as a monitoring tool.
- Although only a small number of instruments were sampled, results indicate that considerable proteinaceous soiling is still present on most of the instrument's surface even after cleaning.
- 56% of the instruments inspected showed severe contamination in at least one of the sample regions and only 9% displayed low-level contamination.

### **4.3 Surgical instrument protein contamination survey -2003 updated results**

In autumn 2003, the Department of Health sent a series of ready-for-use instrument trays containing a variety of surgical instruments to DECON research groups for analysis of residual protein. The instruments were collected from five anonymous NHS Primary Care Trust SSDs to investigate the efficacy of 'in-place' cleaning procedures. The results from that survey have been updated.

#### **4.3.1 Analysis of protein levels by ELISA**

Dr Mark Sutton, at the HPA, carried out an assessment of total protein load on surgical instruments by a conventional commercial ELISA assay. Instruments were soaked overnight at 60°C in 1% sarkosyl and 6M urea. Overall 17% (35/206) of the instruments contained over 200 µg of total extractable protein, but higher percentages were achieved for some classes of instruments, such as retractors and scissors. It was noted that instruments used in tonsillectomies had higher amounts of protein bound to them.

#### **4.3.2 Analysis of protein levels by total chemical analysis**

Professor Robert Baxter's team at Edinburgh University analysed surgical instruments for total residual protein contamination, using total chemical extraction. This was accomplished by stripping the instruments surface with hydrogen chloride to completely hydrolyse the protein, followed by total amino acid analysis. This method achieved a total protein detection limit of 10 µg. The results showed that there was variation between instruments in the level of contamination detected. The protein mass for forceps ranged from 5 to 960 µg. Tonsillectomy instruments were all shown to have a protein mass above 500 µg and a simple retractor contained nearly 1 mg of protein. However, the amount of protein contamination on the instruments did not correlate with the instrument's complexity.<sup>16</sup>

#### **4.3.3 Analysis of protein levels by episcopic differential contrast microscopy**

Professor Keevil's group at the University of Southampton has undertaken a double blind study of surgical instruments using the EDIC/EF method. The group examined nine sets of instruments (average 40/tray). In total 260 instruments were assessed for contamination; these ranged in size and shape and included flexible and rigid endoscopes, tissue forceps and Zoellner suckers. All the instruments were visually inspected on arrival by a researcher familiar with the different instrument layouts and were scored for their cleanliness between 0 (no visible contamination) and 2 (large amounts of visible contamination) in all of the defined sample regions.

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<sup>16</sup> Baxter RL, Baxter HC, Campbell GA, Grant K, Jones A, Richardson P & Whittaker AG. (2006). Quantitative analysis of residual protein contamination on reprocessed surgical instruments. *J Hosp Infect*, **63** (4): 439-44.

The instruments were stained with SYPRO Ruby and visualised by EDIC/EF. All of the instruments were examined at multiple sample points over their surface and scored by the visual contamination index described in section 4.2. Of the instruments inspected visually, 37% (n=75/202) showed a low level of contamination with a mean score of <1 and only 4% (n=8/202) showed high levels of contamination (mean score >1.5). The results from the EDIC/EF microscopy analysis indicated that 66% of all the instruments inspected showed severe contamination (>4.2 µg protein/mm<sup>2</sup>), 27% were moderately contaminated (0.42-4.2 µg protein/mm<sup>2</sup>), and only 7% displayed low-level soiling (classes 0-420 ng protein/mm<sup>2</sup>).

Within the instrument subgroups examined, 41% (n=49) of the hinged and 31% (n = 26) of the simple instruments had visual assessment scores of <1 (0-42 ng/mm<sup>2</sup>). Statistical analysis of these data sets showed that the amount of protein contamination on the instruments did not correlate with the instrument's complexity. These results support those of the Edinburgh group (section 4.3.2.).

Some of the instruments appeared heavily soiled when observed using EDIC microscopy. This soiling was not proteinaceous as it did not stain with SYPRO Ruby (SR). The soiling frequently appeared crystalline in nature and may have consisted of deposits remaining from the use of detergent or enzymatic cleansers in the washer-disinfectors (WDs). These deposits could not be observed by eye, perhaps suggesting that their occurrence may have been more widespread than hitherto realised.

Image analysis of a composite of the EDIC/EF images clearly showed the SR-positive proteinaceous material adhering on the surface of these non-proteinaceous crystalline deposits. Many of the adjacent areas displayed relatively little SR-positive material, suggesting that either: (i) protein binds preferentially to the crystalline deposits compared to stainless steel; or (ii) the protein is more difficult to remove from the crystalline deposits during the cleaning process; or (iii) crystalline deposition may occur in inaccessible regions of the instrument that are difficult to clean. If they are found to occur commonly then consideration must be given to the formation of cleaning agents used in WDs and to the way in which WDs are operated with respect to temperature and rinse cycles

Although this technique requires further development before it can be applied to every instrument passing through an SSD, its simplicity and speed would allow it to be used as a 'spot check' to confirm that standards of cleanliness are being maintained. In addition, since the SYPRO Ruby fluorescent probe binds only to protein, the removal of such contamination would also remove this marker and have no effect on subsequent sterilisation methods.<sup>17</sup>

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<sup>17</sup> Lipscomb IP, Sihota AK & Keevil CW. (2008). Comparison between visual analysis and microscope assessment of surgical instrument cleanliness from sterile service departments. *J Hosp Infect*, **68**: 52-58.

#### **4.3.4 Analysis of protein levels by derivatisation of simple amines with o-phthaldialdehyde**

Professor Perrett's group used the OPA/NAC assay to quantify protein contamination on a series of ready to use instruments collected from five hospitals. Noticeable differences in the levels of protein contamination were noted between the five hospitals with the maximum level of protein contamination reaching 490 µg of protein per instrument. Instruments with high residual protein contamination included tonsillectomy snares and tweezers that had noticeable tissue lumps at the base. In some cases, where pieces of tissue were clearly visible, the method grossly underestimated the amount of protein present on the instrument. This was thought to be because not all of the protein was solubilised.

This led to a study in which residual material from washed instruments were filtered through a 0.45 µm filter and particulates weighed. This assay showed that residue weights ranged from not detectable to 7500 µg, but no correlation was found between the amount of protein present and the complexity of the instrument.

In addition, Barts' Hospital Central Sterile Services Department supplied two series of instruments to be examined by Professor Perrett's group. One series had passed Quality Control and was to be passed to the theatres for use; the other series had failed the inspections. Both sets had been washed in Solid Metal Pro and were subjected to a further intensive wash using 1% Decon in the laboratory. The types and sizes of the instruments varied considerably, hence a comparison of the raw data is difficult. However, the highest levels of protein that could be removed ranged from 24-116 µg per instrument: surprisingly there was no real difference between passed and failed groups.

Studies have also been made by Professor Perrett's group in conjunction with Dr Andrew Smith and Sharon Letters (general dental practitioner in Glasgow) on the residual protein found on re-useable dental files (reamers). Examination of 220 randomly collected files supplied by dental practices in Scotland found residual protein levels averaged 9.2 µg (range 0.8 – 42 µg) and roughly correlated with the gauge of the file. The majority of the files were also visibly contaminated, but the chemical nature of the contamination is at present not known.<sup>18</sup>

Initial experiments to adapt the OPA/NAC protein assay for *in situ* determination of proteins have been carried out. Although protein could be detected *in situ* on stainless steel using a scanning fluorimeter, sensitivity was reduced significantly due to reflection of the excitation light from the surface of the steel.

#### **Key findings**

- All groups showed instruments designated 'ready for use' were contaminated with proteins.

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<sup>18</sup> Smith A, Letters S, Lange A, Perrett D, McHugh S & Bagg J. (2005). Residual protein levels on reprocessed dental instruments. *J Hosp Infect*, **61**(3): 237-41.

- Analysis by ELISA found 17% of the instruments contained over 200 µg of total extractable protein.
- EDIC analysis found 66% of instruments showed severe contamination (>4.2 µg protein/mm<sup>2</sup>).
- Total chemical extraction by HCl showed all tonsillectomy instruments were contaminated with >500 µg protein.
- OPA/NAC assay found maximum level of protein contamination reaching 490 µg of protein per instrument.
- Total chemical analysis showed all tonsillectomy instruments were contaminated with more than 500 µg protein contamination and a simple retractor contained nearly 1 mg of protein.
- Noticeable differences in the levels of protein contamination on instruments were noted between the five hospitals.
- All analytical methods found tonsillectomy snares and retractors had high levels of protein contamination.
- Contamination of instruments was not all proteinaceous. EDIC/EF showed crystalline nature of deposits may be artefacts of the detergent or enzymatic cleansers in the WDs.

#### ***4.4 Quality control in CSSD washers and instrument design considerations***

Studies were performed by Professor Perrett's group on the quality control of the washing systems in the CSSD. The washing systems using unbuffered Solid Metal Pro had wash baths with pH varying by up to 1 pH unit during a half-day shift. The conductivity of the bath liquors varied in a similar manner.

A number of instruments obtained at random from the CSSD were examined visually for contamination by dismantling them. Instruments such as forceps were found to show gross contamination in certain areas, such as moving joints, and instruments with lumens were found to have considerable material within the lumens. However, initial chemical tests have not been able to confirm the presence of protein in these areas, where the major build-up may simply be 'rust'.

##### ***Key findings***

- Up to 60 mg of tissue can contaminate even simple surgical instruments during use.
- Instruments obtained from CSSD regardless of their visual appearance can still contain up to 1mg of protein.
- It is suggested that a monomolecular layer of various proteins forms on the surface of stainless steel and is difficult to remove under CSSD conditions.

- Similar considerations apply to dental instruments.
- Instrument design could be considerably improved to remove areas that cannot be cleaned, although it is probably impracticable to eliminate all such areas.

#### ***4.5 Evaluation of cleaning methods on protein contamination of dental instruments***

A collaborative project with the HPA, Glasgow Dental Hospital, and Barts' Medical School has evaluated the efficiency of three instrument cleaning methods used in general dental practice (GDP): manual cleaning only, manual plus ultrasonic cleaning and benchtop washer disinfectors. The study also looked at washer disinfectors (WD) located in SSDs.

A range of reprocessed instruments used in clinical practice from both GDPs and SSDs were examined for presence of residual risk materials using a combination of visual score and a sensitive protein assay based on o-phthaldialdehyde/N-acetyl cysteine as the fluorescent reagent with reference to a standard curve determined using BSA.

No significant differences were present between the manual only, SSD WD or benchtop WD cleaning processes. However, significant differences were observed between each of these processes and the combined manual-ultrasonic cleaning process, where larger residual protein deposits remained. Regression analysis showed no positive correlation between visual scoring and residual protein ( $p > 0.05$ ). Except for the SSD WD, all methods showed large variations between different instruments of the same type decontaminated by the same method.

Cleaning efficiencies of the different processes varied considerably and demonstrated the manual plus ultrasonic cleaning process to be significantly less efficient than the other three processes in terms of residual protein, though the proportion of residual human protein was not examined. Visual scoring was not a reliable measure of cleanliness. These issues require further investigation but may have implications for the type of cleaning processes used within GDPs and SSDs.



## 5 DEVELOPMENT OF DECONTAMINATION PROTOCOLS

Several groups have examined how different decontamination protocols (e.g. detergent washing, autoclaving, sonication), together with both physical (e.g. pH, temperature) and biological factors (e.g. TSE strain, animal model), may influence TSE inactivation.

The most sensitive methods for assessing TSE inactivation use animal assays, providing a quantitative measure of TSE infectivity. These assays are based on serial dilutions of sample but incubation period measurements can sometimes be used if no confounding factors have been identified.

These studies are not straightforward for a number of reasons. Both the effective titre (the ability of a TSE source to infect a recipient) and the incubation period of the disease are influenced by a number of factors. Incubation period can differ independently of effective titre so, for example, titrations in two mouse PrP genotypes can give similar values but the incubation periods may differ greatly.

The factors influencing titre and incubation period are summarised in Table 4. It is important to distinguish between these two parameters: the most rigorous method of doing so is to perform titrations to end point and then to compare the dose response curve of the titrations, after adjusting for differences in effective titre (or dose).

**Table 4. Conditions influencing disease parameters**

CONDITION	Incubation period	Effective titre
Strain of agent	+	+
Dose of agent	+	+
Infecting tissue	+	+
Status of infecting tissue	+	+
Route of infection	+	+
Species of donor (including PrP genotype)	+	+
Species of recipient	+	+
Status of recipient (particularly immune system)	+	+
Age of recipient	+	-
Sex	+	-
Genotype recipient (identified factors include MHC types)	+	+
PrP genotype of recipient	+	+

The limit of detection is determined by the lowest dilution assayed. The clearance of an experiment (i.e. the reduction in titre that can be demonstrated for a process) is determined by the starting titre of the untreated sample and the limit of detection. Only quantitative reductions in TSE infectivity can be measured. There can be no measurement or guarantee of absolute sterility. It should be borne in mind that samples with no detectable infectivity may be just below the limit of detection: there is no qualitative guarantee of removal of infectivity.<sup>19</sup>

The species barrier is a statement of the increase in difficulty of transmitting a TSE infection from one species to another, compared to the transmission properties within a species. Operationally it is defined by the difference in effective titre and of incubation period between the primary passage into a new species and the effective titre and incubation period of subsequent passes. For example, BSE transmits to C57 BL mice with an incubation period of about 450 days and a titre of  $10^3$  to  $10^5$  per gram, but at subsequent passages has a titre of about  $10^9$  and an incubation period of 180 days. Factors influencing species barriers are poorly understood but include changes in agent properties and poor uptake of agent by some tissues at primary passage (e.g. by brain).

### ***Mechanisms of TSE Inactivation***

TSE infectivity is generally considered to be chemically stable, resistant to radiation, and relatively thermostable, especially that of BSE-derived TSE strains. It has been shown that TSE infectivity can however be destroyed by extreme treatments such as combustion (e.g. incineration), chemical oxidation (e.g. sodium hypochlorite), and hydrolysis at high pH. Nonetheless, TSE infectivity may have different inactivation properties depending on prior treatments, such as dehydration, which could make TSE infectivity more resistant to inactivation.

Techniques which are likely to be milder, probably denature TSE agents, and include heat when the sample is hydrated and exposure to denaturing chemicals (e.g. strong detergents, chaotropes). Variables which may affect the success of treatments include biological properties such as TSE agent strain, host genotype, and tissue type. The dynamics and kinetics of the inactivation process may be affected by time of exposure, concentration of inactivating chemicals, temperature, and pH.

Ideally, inactivation protocols should be tested on the most resistant TSE models available if they have been identified. The thermostability properties of a range of TSE models have been characterised to some extent and form a basis for the selection of appropriate models. However much less information is available for other mechanisms of inactivation, e.g. with high pH or strong detergent. Where such information is lacking it would be desirable to compare inactivation in a range of TSE models. Dehydration and other protein fixation treatments may compromise inactivation protocols. The efficacy of inactivation

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<sup>19</sup> Somerville RA. (2007). TSE models and their use in assessing TSE agent inactivation. Presentation to ESAC-Pr, London 2007.

protocols should be quantified and compared to the required degree of TSE inactivation, established by risk assessment.<sup>19</sup>

## **5.1 Factors affecting TSE infectivity**

The effect of temperature and pH on TSE infectivity using animal assays has been studied by Dr Robert Somerville at the Institute of Animal Health in Edinburgh. Since the biochemical properties of TSE infectivity do not always correlate with those of PrP<sup>Sc</sup>, the effect of temperature and pH was assessed for TSE infectivity and not just for protein removal.

### **5.1.1 Effect of temperature and pH on TSE infectivity**

Studies have shown that heat inactivation occurs rapidly immediately after exposure to high temperatures but the rate of inactivation slows with time, following second order kinetics. This leads to the hypothesis that heat may inactivate infectivity by the dissociation of two components, but may also promote a protective reaction. At much higher temperatures, protein is destroyed. Experiments demonstrated that the heat inactivation of infectivity is dependent on temperature, TSE strain and hydration status, but not on PrP genotype.

Experiments at high pH values (above pH 12) also show that inactivation follows second order kinetics. There is a synergistic effect of pH and temperature, specifically increasing pH above pH 11 reduced the thermostability of the three TSE strains studied (22C, ME7 and 301V).

Experiments on tissue which had been previously heated to temperatures which inactivate one TSE strain (22C) but not another (22A) showed that whereas infectivity survived in the case of 22A, PrP<sup>Sc</sup> which is normally resistant to protease digestion became susceptible to digestion after heating and similarly for both 22A and 22C sources. These experiments demonstrate the properties of PrP<sup>Sc</sup> do not necessarily match those of TSE infectivity and therefore PrP<sup>Sc</sup> cannot be used as a surrogate of TSE infectivity.<sup>20</sup>

### **5.1.2 Effect of heat on TSE infectivity**

Heat inactivation under hydrated conditions appears to follow second order kinetics, hence there is little effect of time after initial exposure and the inactivation curve is biphasic with respect to time. An experiment in which 10 TSE models, comprising five mouse passaged TSE agent strains in two PrP genotypes of mouse showed a major difference in resistance to autoclaving between TSE strains, which was little affected by PrP genotype. These results reflect the fundamental thermostability properties of TSE.

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<sup>20</sup> Somerville RA, Oberthür RC, Havekost U, MacDonald F, Taylor DM & Dickinson AG. (2002). Characterisation of thermodynamic diversity between TSE agent strains and its theoretical implications. *J Biol Chem*, **277**: 11084-11089.

### **5.1.3 Effect of pH and heat on TSE infectivity**

Studies have demonstrated that there is a synergistic effect of heat and high pH. In one experiment there was a significant reduction in TSE infectivity with time at pH 13, but not at pH 12 or lower. Preliminary analysis indicates differences in sensitivity to high pH between TSE strains at 20°C, which correlates with the differential survival of TSE infectivity in hypochlorite. High temperatures reduce the pH of TSE inactivation. It is possible that partial inactivation by high pH (e.g. between pH 12 and 13 for 301V) may stabilise TSE agents, e.g. increase resistance to autoclaving.

## **5.2 Autoclaving**

### **5.2.1 Novel surgical instrument decontamination procedures - Validation of a novel surgical instrument steriliser**

Laboratory tests with combinations of alkali and pressurised steam have previously been proven to be very effective with TSE agents. These two projects, led by Robert Somerville and Karen Fernie aim to study the effect of such treatments on infectivity dried onto the surface of surgical stainless steel.

The development of novel surgical instrument decontamination procedures using a range of combinations of lower temperature and lower molarities of hydroxide on PrP<sup>Sc</sup> are underway. Combinations which prove the most effective at this stage will then be examined for infectivity by bioassay in mice.

These experiments rely on the recovery of persistent PrP and/or infectivity from the surface of the instrument following decontamination attempts. Initial studies had to examine the recovery efficiency. The recovery consistency is being increased at each stage but remains the limiting factor in determining the efficacy of the various heat hydroxide combinations on removing PrP<sup>Sc</sup> from the surface of the test instruments. Despite recovery inconsistencies, these experiments have demonstrated that PrP which has been hydroxide treated, can be detected and a reduction in the amount of PrP present using dilute solutions of NaOH has been demonstrated. If the variability in recovery is not resolved, the experiments may have to be continued using stainless steel samples implanted intracerebrally to test the efficacy of the heat/hydroxide decontamination procedures.

PrP has also been successfully detected directly from the surface of a test instrument onto which brain material had been dried. This method may have the potential to look at the presence of PrP on real instruments.

These experiments are also providing fundamental information about the properties of PrP under different conditions. This information may be useful when determining how PrP and perhaps infectivity dried on to instruments will behave following unsuccessful surgical instrument decontamination attempts.

The American company WR<sup>2</sup> has developed a system that combines these procedures in equipment designed for use with TSE-contaminated surgical instruments. A series of experiments have been designed to validate this system. These include NaOH and 150°C; NaOH and 100°C, NaOH and

121°C; KOH and 100°C with ultrasonics; and NaOH and 100°C without ultrasonics. The design of a laboratory facsimile of the WR<sup>2</sup> equipment for these experiments will be determined by the success of the recovery protocols and whether changing to using intracerebral implants is required.

### ***5.2.2 Assessment of damage by alkali autoclaving***

Experiments carried out by Karen Fernie have looked at possible damage caused by combining chemical treatments and high temperatures.

#### ***Surgical instrument material***

To examine the effects of combined NaOH and heat, test “instruments” in the form of surgical stainless steel pieces, 25mm in diameter and 5mm thick, were manufactured by two different surgical instrument manufacturers in exactly the same way as those on the market (Surgical Instruments Engineering Ltd. and Rocket Medical). Four different grades of stainless steel were represented, with one grade being common to both manufacturers. Some of the most common finishes were represented including matt finished, brightly polished, serrated (as on the blades of forceps) and titanium nitride coated which is now widely used. This gave 13 different grade/finish combinations.

These test pieces were subjected to heat/hydroxide decontamination procedures and conventional recycling in a hospital SSD. The stainless steel surface was analysed both before and after treatment. They were examined grossly and photographed, examined for signs of damage by both white light scanning interferometry, a 3D imaging method, and by scanning electron microscopy.

#### ***Experimental protocol***

Using information from Lothian Central Sterilisation Unit, it was estimated that instrument sets may be used and processed as many as 200 times a year. The treatment examined was designed to mimic 96 individual treatments or approximately six months of heavy use. Autoclaving at 121°C for 15 minutes in 2M NaOH completely inactivates infectivity in brain tissue samples infected with several different TSE agents, therefore the test pieces were exposed to 2M NaOH at 121°C for 24 hours, designed to approximate 96 x 15 minute cycles. For comparison, a set of the test pieces were processed conventionally 96 times at the Edinburgh City Hospital Central Sterilisation Unit. Several pairs of new artery forceps were treated alongside to ensure that the test pieces were representative of real instruments.

Test pieces were examined at two magnifications (x2.5 and x40) using a white light scanning interferometer. At each of the magnifications test pieces were examined in five areas. The mean roughness indices from these five areas were then compared. The results show that the damage (as measured by an increase in the roughness indices) caused by treatment with NaOH and heat, was, in the majority of cases, not significantly different from that caused by conventional recycling methods. It was also noted that a number of the samples which had been reprocessed conventionally appeared to have increased amounts of debris present.

This study has shown that heat/hydroxide combinations may be a practical method for recycling surgical instruments judging by visual appearance and two assessments of surface damage.

### ***5.2.3 Effect of porous load autoclaving at 134°C and 138°C on the inactivation of scrapie and BSE infected brain tissue***

Autoclaves are designed to inactivate infectious organisms by imparting heat energy directly from steam to the surface of the sample. Heat transfer from steam is particularly efficient. The simpler mode of operation is gravity displacement, where steam is fed into the chamber, displacing the heavier air downwards. However, pockets of air may remain in the load. Porous-load autoclaving seeks to purge the air from the autoclave by first evacuating the chamber, then alternately pressurizing and depressurizing the chamber with steam to displace residual air pockets.

Various experiments were performed to study the effect of autoclaving at 134°C and 138°C for 9, 18, 30, and 60 minutes on the infectivity in brain macerate infected with 22A strain of rodent-passaged scrapie, the 301V strain of BSE and the 263K strain of hamster-passaged scrapie.

The 22A strain was the most thermolabile. Autoclaving at 134°C for 9 to 60 minutes left no detectable infectivity and autoclaving at 138°C for 9 to 60 minutes showed only a small amount of infectivity. 301V was the most thermostable. Infectivity was found in up to 100% of the animals injected following both temperatures for up to 60 minutes. There were significantly more animals positive for disease in the 138°C groups than the 134°C groups.<sup>21</sup>

### ***5.2.4 Autoclaving at 134°C and 137°C on the inactivation of BSE, sporadic CJD, and vCJD-infected brain tissue and peripheral tissues***

Karen Fernie has work in progress looking at the effectiveness of heat sterilisation at 134°C and 137°C for 3 minutes, which are the conditions used to sterilise the majority of surgical instruments in SSDs.

Tissues being investigated include vCJD infected brain tissue exposed to both temperatures and peripheral tissues (spleen, tonsil, lymph node) exposed to 134°C. Sporadic CJD, BSE and 301V tissues have also been included for comparison. An autoclaving experiment with BSE and related sources has shown that after autoclaving at 134°C or 137°C for 3 minutes, the titre was reduced by an average for BSE of 1.6 log ID<sub>50</sub>, (2 samples, vCJD = 2.8 log ID<sub>50</sub>), (4 samples and 301V = 3.5 log ID<sub>50</sub>). These and other data show that BSE and related TSE sources have higher thermostability properties than other TSE sources and in particular they exhibit survival of substantial amounts of infectivity after autoclaving. Varying the conditions of autoclaving

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<sup>21</sup> Fernie K, Steele PJ, Taylor DM & Somerville RA. (2007). Comparative studies on the thermostability of five strains of transmissible-spongiform-encephalopathy agent. *Biotechnol Appl Biochem*, **47**: 175-183.

has little effect on the degree to which TSE agents were inactivated. However dehydration and tissue fixation can markedly increase resistance to heat inactivation perhaps because the size of heat-resistant subpopulations are increased by some physical or chemical treatments.

### **Key findings**

- The alkali heat combinations do not appear to increase damage to instruments, under the test conditions examined.
- TSE infectivity (301V) appears to be stable up to pH 11 and 80°C (or above). At pH 12 it is stable at 20°C but titre is reduced at 60°C. Very little infectivity is recovered at pH 13, and none so far at 80°C or above.
- At pH 11 and 75°C (or above) PrP<sup>Sc</sup> is susceptible to proteinase K digestion, and at pH 12 and 75°C and above, PrP<sup>Sc</sup> appears to be degraded, even without proteinase K.
- Preliminary results from the autoclave assessment indicate that vCJD is at least as insensitive to heat as BSE.
- These autoclaving regimes may compromise inactivation of TSEs because the agents may be dehydrated and residual infectivity stabilized.

### **5.2.5 Effect of dry heat exposure at 200°C on infectivity TSE agents**

To determine the effect of dry heat treatment on TSE inactivation, brain samples from three TSE models (ME7 in SV mice, 301V in VM mice and 263K in LVG hamsters) were heated at 200°C in a 'dry heat' oven for 20 or 60 minutes, rehydrated and titrated by serial dilution in mice.<sup>22</sup> Titres were reduced by approximately 3 log ID<sub>50</sub> (infectious dose) after heating of all three samples for 20 minutes, with little additional loss after 60 minutes (0–0.7 log ID<sub>50</sub>). There was a marked prolongation of the incubation period of heated ME7 samples compared with the same equivalent doses in the unheated control ME7 sample, the effect being significantly greater after 60 minutes than after 20 minutes. There was only a small shift in the 263K dose-response curve on increasing the heating time and no effect on dose-response curves of heated 301V. The survival properties of TSE infectivity exposed to dry heat were not strain-specific, unlike the differences in heat-inactivation properties observed in aqueous solution.<sup>23</sup> However, the interaction with the host of the surviving subpopulation of TSE agent was altered in a TSE strain-specific fashion.

### **Key findings**

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<sup>22</sup> Fernie K, Steele PJ, Taylor DM & Somerville RA. (2007). Comparative studies on the thermostability of five strains of transmissible-spongiform-encephalopathy agent. *Biotechnol Appl Biochem*, **47**:175-183.

<sup>23</sup> Somerville RA, Oberthur RC, Havekost U, MacDonald F, Taylor DM & Dickinson AG. (2002). Characterisation of thermodynamic diversity between TSE agent strains and its theoretical implications. *J Biol Chem*, **277**: 11084–11089.

- Although TSE infectivity was reduced after heating to 200°C in a hot air oven, substantial amounts of infectivity remained.
- Unlike wet heat inactivation, no TSE strain-dependent differences in infectivity were observed.
- Dry heat substantially prolonged the incubation periods of mice infected with ME7 compared to unheated controls depending on exposure time, whereas there was little or no effect for 301V or 263K.

## **5.3 Efficacy of current NHS procedures for protein removal**

### **5.3.1 Assessing standard NHS reagents by ELISA**

The indirect enzyme-linked immunosorbent assay (ELISA), developed by Dr Denise Dear, was used to assess the efficacy of rPrP removal using: guanidine hydrochloride; an RCA etch - based on ammonium hydroxide (NH<sub>4</sub>OH) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), commonly employed in the semiconductor industry; and sodium hydroxide (NaOH) solutions. There was a significant difference between washes and between the amounts of protein removed, depending upon the length of time the protein had been allowed to adsorb to the surgical steel. However, none of these reagents alone appeared to be capable of removing significant quantities of rPrP from surgical steel, as assessed by the ELISA. The effectiveness of reagent mixtures at removing prion protein still remains to be assessed.

Epitope mapping or mis-mapping by antibody employed in the ELISA technique may be the reason for anomalous results.

In the presence of mixed proteins the masking of epitopes by other proteins or competition for binding sites on the surface, i.e. the Vroman effect, could be creating the problem in this ELISA.

### **5.3.2 Assessing standard NHS reagents by MARS**

Studies were carried out by Dr Raj Sethi on stainless steel and glass using MARS and supported by X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM). To determine the effectiveness of NHS reagents at removing prion protein, steel and glass samples were incubated for 48 hours with rPrP (100 µg/ml) mixed with a complex mixture of proteins, such as ovine brain homogenate (with a total protein concentration of 20 mg/ml) and then washed with different wash solutions.

A 3M solution of guanidine hydrochloride was shown to remove 25% of adsorbed proteins from steel. This treatment was more effective at removing bound proteins from glass (47%). Analysis was confirmed by XPS.

The NH<sub>4</sub>OH and H<sub>2</sub>O<sub>2</sub> RCA etch was found to remove 80% of bound proteins, as shown by XPS. However, the treatment was also shown to attack the glass surface.



Stainless steel and glass exposed to a solution of 1M NaOH at 100°C for 60 minutes removed bound rPrP<sup>C</sup> (100 µg/ml) and complex mixtures of proteins, such as those found in ovine brain homogenates (20 mg/ml). XPS analyses of these surfaces revealed the presence of carbon contamination. It is postulated that this may have been picked up from the alkali solution. However, this cleaning process caused a disruption of the surface of steel, manifested as an increase in surface roughness of 33%, determined by atomic force microscopy (AFM).

Steels (430 and 316) washed with 1M boiling NaOH for up to 30 minutes showed up to 75% increase in surface roughness before the polishing effect took place (after 60 minutes) as determined by AFM. These surface modifications of the steels allowed an increased adsorption (confirmed by XPS) of proteins such as those in mouse serum (10 mg/ml) and were linked with increased wettability and surface energies, as determined by contact angle measurements on these treated surfaces.

### **Key finding**

- These treatments were relatively inefficient at removing proteins bound to steel and glass surfaces and could cause some surface damage.

### **5.3.3 Effect of hypochlorite on TSE inactivation**

The use of high concentration hypochlorite solutions (also known as chlorox, it is the major inactivating component of common bleach) has been considered an effective way of achieving a high degree of TSE inactivation. It is considered by some to be the most effective chemical disinfective agent, the only recommended alternative being very high concentrations of NaOH, i.e. high pH solutions (pH > 14). However in some situations TSE inactivation with hypochlorite is incomplete.

The first major study of hypochlorite inactivation of TSEs by Kimberlin *et al.*, (1983),<sup>24</sup> showed that exposure to a hypochlorite solution containing 13,750 ppm available chlorine for 30 minutes was effective in inactivating two mouse passaged scrapie agents, 22A and 139A. This result formed the basis for the recommendation of a 1 hour exposure to sodium hypochlorite containing 20,000 ppm available chlorine as an effective decontamination method. Another experiment carried out by Brown *et al.*, (1986)<sup>25</sup> showed a more concentrated solution of hypochlorite was not completely effective. In their experiment, despite destroying all detectable 263K/hamster scrapie, guinea pig passaged CJD was only reduced by 3.3 logs following exposure to 25,000 ppm hypochlorite. In two experiments, Taylor *et al.*, (1994)<sup>26</sup> examined the

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<sup>24</sup> Kimberlin RH, Walker CA, Millson GC, Taylor CM, Robertson PM, Tomlinson AH & Dickinson GA. (1983). Disinfection studies with two strains of mouse-passaged scrapie agent. Guidelines for Creutzfeldt-Jakob and related agents. *J Neurol Sci*, **59** (3): 355-369.

<sup>25</sup> Brown P, Rohwer RG & Gajdusek DC. (1986). Newer data on the inactivation of scrapie virus or Creutzfeldt-Jakob disease virus in brain tissue. *J Infect Dis*, **153**(6): 1145-1148.

<sup>26</sup> Taylor DM, Fraser H, McConnell I, Brown DA, Brown KL, Lamza KA & Smith GR. (1994). Decontamination studies with the agents of bovine spongiform encephalopathy and scrapie. *Arch Virol*, **139**(3-4): 313-326.

effects of sodium hypochlorite on BSE samples. The results showed that there was no detectable infectivity after exposure to solutions of sodium hypochlorite containing between 8,250 and 16,500 ppm available chlorine for times ranging from 30 minutes to 1 hour. However, the effective starting titres of these primary BSE samples were only  $10^{3.6} - 10^{4.1} \log_{10} \text{ID}_{50}/\text{g}$ . It is important to note that formulations of the hypochlorite solutions used in previous studies were no longer available. The experiments carried out in the current studies used a general supply of sodium hypochlorite which is used routinely in the laboratory for decontamination procedures.

In a recent experiment by Fernie and Somerville, three TSE strains were exposed to chloros. Brain homogenates were treated with varying concentrations of sodium hypochlorite for 30, 60 or 90 minutes. No infectivity could be detected after treatment of the 22C TSE strain except at the lowest concentration of hypochlorite, but infectivity could be readily detected after treatment of ME7, with all animals succumbing, even after treatment with the highest concentration of hypochlorite. The 301V strain exhibited intermediate properties. Increasing the concentration of hypochlorite and the length of exposure increased the degree of inactivation to a modest degree. These results demonstrate that hypochlorite treatment reduced infectivity amounts but did not fully inactivate. There was a notable difference in sensitivity of the three TSE strains used. Although there is considerable variation, there is a trend in the 301V model for increased inactivation with respect to both time of exposure and concentration of hypochlorite, although the effects were relatively small.

The chloros was used without adjustment for pH or other adjustments to the chemical milieu, as is the normal practice when performing routine decontaminations. Commercial hypochlorite solutions are sold in various formulations which may vary in pH and in other components included to stabilise the solution, all of which may affect activity. Solutions are in equilibrium between the hypochlorite ion and uncharged hypochlorous acid. Lowering the pH to near pH 7 shifts the equilibrium from hypochlorite to hypochlorous acid. Although hypochlorous acid is less stable it is more reactive, hence the oxidising effect of chloros is optimal near neutral pH.

### ***Key findings***

- These initial studies suggest that sodium hypochlorite may not be as reliable in removing TSE infectivity as previously thought.
- Some evidence that the effect of hypochlorite is strain specific.

#### ***5.3.4 Effect of guanidine hydrochloride on TSE infectivity***

Dr Robert Somerville has also carried out experiments to look at the effect of guanidine hydrochloride and temperature on TSE infectivity. The results show a trend towards monophasic inactivation with respect to both guanidine concentration and time. There is no detectable difference in susceptibility to guanidine between the three TSE models tested.

## **5.4 Removal of protein with detergents**

### **5.4.1 Assessing the effect of detergents on protein removal by ELISA**

The indirect ELISA, developed by Dr Denise Dear was used to assess the efficacy of rPrP removal by a variety of detergent washing solutions, including those used in NHS WDs (e.g. sodium *n*-dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), deoxycholate, APO-12, BRIJ 35, and Zwittergent 3-16). The detergents removed different amounts of protein, depending upon the length of time the protein had been allowed to adsorb to the surgical steel. None of these detergents alone appeared to be capable of removing significant quantities of rPrP from surgical steel, as assessed by ELISA.

The ELISA on stainless steel discs was also used to assess the effect of SDS combined with NaOH on protein removal. It was found that 2M NaOH and 1% SDS could remove up to 80% of rPrP.

#### **Key findings**

- None of the detergents tested appeared to be capable of removing significant quantities of rPrP from surgical steel, as assessed by ELISA.
- In combination, 2M NaOH and 1% SDS could remove up to 80% of rPrP from stainless steel.

### **5.4.2 Assessing the effect of detergents on protein removal by XPS**

Dr Raj Sethi has assessed the effectiveness of different detergents on prion protein removal, from steel and glass using XPS.

Steel samples were incubated with rPrP (100 µg/ml) mixed with a complex mixture of proteins such as ovine brain homogenate (20 mg/ml) for 48 hours and subsequently washed with different detergent solutions, including SDS (3%, w/v), CTAB, deoxycholate, APO-12, BRIJ 35, and Zwittergent 3-16. The concentrations of the detergent solutions were 10 times above the critical micelle concentration and their pH ranged between 6 and 8. XPS showed that CTAB removed 45% whereas other reagents had no significant effect.

Similar experiments conducted with glass employed for optical components in surgical instruments showed that the amount of protein adsorbed to glass was approximately 70% higher than steel. However, all the washing reagents were more effective in removing proteins from glass compared to steel. SDS (3%, w/v), BRIJ 35, Zwittergent 3-16, deoxycholate, and CTAB removed respectively 75%, 28%, 23%, 20%, and 8% of proteins as confirmed by XPS.

#### **Key findings**

- XPS showed that CTAB removed 45% of prion protein from stainless steel whereas other reagents had no significant effect.
- All washing reagents were more effective in removing proteins from glass compared to steel.

- SDS removed 75% of proteins from glass whereas CTAB removed 8%.

#### **5.4.3 Assessing the effect of detergents on protein removal by EDIC/EF**

Decontamination studies by the Southampton group were also undertaken to determine the efficiency of a commercially approved CSSD detergent (Hamo 54) in removing residual protein. The results showed that after 90 minutes with a constant flow of the detergent solution, most of the residual protein had been removed. However, the average recommended detergent wash time in a CSSD is 5 minutes, which according to this work would only remove 5% of the protein.

#### **Key finding**

- 90 min with a constant flow Hamo 54, removed most residual protein but this is more than the average recommended detergent wash time in CSSD of 5 min, which according to this work would only remove 5% of the protein.

#### **5.4.4 Assessing the effect of detergents on protein removal by the OPA/NAC assay**

Studies by Professors Perrett and Jeffries at the Barts' and London School of Medicine and Dentistry were designed to evaluate current decontamination procedures for surgical instruments.

Studies on the quantitative removal of model proteins by the o-phthalaldehyde / N-acetyl cysteine (OPA/NAC) assay were performed with the following proteins: BSA, collagenase, cytochrome c, fibrin, fibrinogen, gamma globulin, haemoglobin, and lysozyme. In addition, simple studies with whole human blood were also performed. In all cases, proteins were dried onto stainless steel blades at 37°C for 1 hour prior to washing. Studies were conducted with a number of regular laboratory detergents as well as water. In the case of the model proteins, all could be removed to levels higher than 99.9% with dilute solutions of laboratory detergents, such as Decon 90, with a sequence of three washings - the majority being removed in the first wash. In the case of dried whole blood, 95% of proteins were removed with one wash in water alone and 99.5% were removed with just three washes in water. These studies proved the principle of the approach and the utility of the OPA/NAC assay.

#### **In vitro residual protein experiments**

In order to mimic the worse case scenario, tissue was dried onto all surfaces at 37°C. The contaminated areas of the instruments were then immersed in suitable detergent solutions, including those from the CSSD and sonicated for appropriate periods of time. Two or four changes of detergent solution were employed. The amount of protein in each solution was determined using the OPA/NAC method and the total amount of protein washed off was calculated. Results from a series of such studies are shown in Table 4.

**Table 4. Efficiency of total protein removal from stainless steel blades**

Detergent	Percentage protein remaining after 4 washes		
	BSA	Kidney	Brain
Solid Metal Pro (1.8 mg/ml)	n.d.	n.d.	n.d.
Solid Metal Pro (1.0 mg/ml)	n.d.	10%	9%
Enzyme Cleaner (1%)	n.d.	3%	n.d.
Scan Clean (1%)	26%	38.5%	18%
NaOH (1 M)	n.d.	15%	6%
Decon 90 (1%)	n.d.	24%	12%

n.d. – not detectable

The most efficient washing solutions were found to be Solid Metal Pro, a CSSD detergent, which consists mainly of NaOH plus various silicates. All detergents (except Scan Clean) readily removed standard protein solutions. Removal of tissue contaminants was not as effective. Kidney tissue was found to be the most difficult to remove. Solid Metal Pro at 1.8mg/ml and 1% enzyme cleaner were most efficient at removing brain tissue.

The OPA/NAC procedure was shown to be reasonably consistent given the difficulties associated with reproducibly contaminating the surface in the first instance.

### **Key findings**

- Methods for the studies of *in vitro* removal of protein have been optimised.
- Adsorbed proteins and protein mixtures can be readily removed using a variety of detergents, but tissues are much more difficult.
- Solid Metal Pro and an enzyme cleaner were considered to be the most efficient at removing protein from stainless steel.
- In the case of the model proteins all could be removed to levels higher than 99.9% with dilute solutions of laboratory detergents, such as Decon 90 after 3 washes.
- In dried whole blood 95% of proteins were removed with one wash in water alone.
- In dried whole blood 99.5% of proteins were removed with just 3 washes in water.

#### **5.4.5 Assessing the effect of SDS detergent on TSE infectivity**

Dr Robert Somerville also assessed the effect of sodium *n*-dodecyl sulphate (SDS), but on TSE infectivity, not on protein removal.

These experiments demonstrated that SDS had little effect at concentrations below 1%, at pH  $\leq 9$ , on the removal of infectivity in different TSE strains. In addition, a treatment of up to 1% SDS for 30 minutes was found to give the same results for a different host strain (mouse). The combination of SDS, high pH and heat may result in efficient inactivation. SDS (1%) at pH 10 may inactivate TSE infectivity monophasically with respect to temperature, but it may be necessary to examine the state of the sample after treatment. The combination of proteinase K (100  $\mu\text{g/ml}$ ) and SDS for 30 minutes had no effect on the removal of TSE infectivity. However, the use of more powerful proteases may be more effective.

### ***Key finding***

- SDS alone was inefficient in removing proteins, but better results were obtained in the presence of heat and high pH.

### ***Future work***

- These initial results indicate that a combination of heat, high pH and SDS is worth further investigation and that the SDS experiments should be repeated with more powerful proteases and on fresh brain and freeze-dried samples.
- A paper published by Peretz *et al.*, (2006)<sup>27</sup> strongly suggests that the use of SDS should be examined further.

## ***5.5 Proteolytic degradation of TSE Agents***

Genencor International Inc. has collaborated in and partly funded this HPA (Porton Down) project to develop methods for the proteolytic inactivation of TSE agents, designed for the decontamination of surgical instruments and animal waste.

### ***5.5.1 Effect of proteases on TSE infectivity***

Initial studies carried out by Dr Neil Raven's group defined thermostable proteases that could eliminate the prion monomer and give approximately a 3 log reduction in the infectious dose by titration of BSE (301V) in VM inbred mice. A combination of modified proteases (identified with potentially improved properties at defined temperature and pH) and alkaline pH has been shown to eliminate all prion immunoreactive material on Western blots of BSE (301V) iMBH. Data from the corresponding mouse bioassay experiments has also shown a reduction in infectious dose of more than 7 logs, based on the titration study. Recent work has shown that MC3 digestion at 60°C and pH 12 reduces infectivity by more than 7 logs, as after 600 days of disease incubation the survival rate is 66%.

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<sup>27</sup> Peretz D, Supattapone S, Giles K, Vergara J, Freyman Y, Lessard P, Safar J, Glidden D, McCulloch C, Nguyen HB, Scott M, DeArmond S & Prusiner SB. (2006). Inactivation of Prions by Acidic Sodium Dodecyl Sulfate. *J Virol*, **80**: 322–331.

Protease treatment could therefore constitute a pre-cleaning stage for TSE decontamination.

### ***Key technical features***

- The best-performing enzyme MC3 is now commercially available as Prionzyme.
- They can remove virtually all bound infectivity.
- They can act at high pH.
- They do not appear to damage steel surfaces.
- The enzymes do not bind to steel surfaces.
- The enzymes have been tested and are not toxic or allergenic.
- Data from the mouse bioassay experiments has shown a reduction in infectious dose of  $\geq 7$  log following MC3 digestion at 60°C and pH 12 for 30 minutes.

### ***Future work***

- The group is planning surgical wire implantation experiments designed to mimic the decontamination of surgical instruments.
- The commercialisation of MC3 as “Prionzyme<sup>TM</sup>” is being progressed.

## ***5.6 Proteolytic degradation of vCJD (Prion Unit)***

### ***5.6.1 Effect of proteolytic enzymes on PrP***

Professor Charles Weissmann at the MRC Prion Unit tested the ability of seven proteolytic enzymes, pepsin, trypsin, bromelain, pronase, subtilisin, papain and proteinase K to degrade prions. The efficacy of the enzymes was determined by incubating them with homogenates of vCJD infected human brain and analysing the reaction mixtures by Western blot analysis, to determine the level of residual immunoreactive PrP. The enzymes were evaluated individually, and in pairs, at a range of temperature and pH values, and additional effects of detergents, lipases and metal ions were examined. A combination of proteinase K and pronase, in conjunction with sodium dodecylsulphate (SDS) was shown to degrade PrP<sup>sc</sup> material from highly concentrated vCJD infected brain preparations to a level below the detection limit of the method, an estimated decrease of 5 logs.<sup>28</sup>

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<sup>28</sup> Jackson GS, McKintosh E, Flechsig E, Prodromidou K, Hirsch P, Linehan J, Brandner S, Clarke AR, Weissmann C & Collinge J. (2005). An enzyme detergent method for effective prion decontamination of surgical steel. *J Gen Virol*, **86**: 869-878.

### **5.6.2 Effect of proteolytic enzymes on infectivity**

The effects of these enzymes alone and in combination with other treatments on infectivity was assessed. Typically, wild type mice succumb to prion disease within 160 to 220 days. This study used a line of transgenic mice that overproduce PrP<sup>C</sup> and exhibit a shortened incubation time of about 65 to 100 days. In order to reproduce the conditions that apply to the sterilisation of prion-infected surgical instruments, mouse prions were adsorbed to surgical steel wires, exposed to the sterilisation treatment and inserted into the brains of sensitive indicator mice that had been monitored for signs of infection. Typically, non-sterilised infected wires caused death within 70 days while treatment with the designated enzyme mix prevented infection in virtually all experiments.

#### **Key findings**

- A combination of proteinase K and pronase, in conjunction with SDS was shown to degrade PrP<sup>Sc</sup> material from highly concentrated vCJD infected brain preparations to a level below the detection limit of the method, an estimated decrease of 5 logs.
- Proteolytic enzyme mix was effective in degrading prions adsorbed onto surgical steel wires and prevented infection in virtually all experiments.
- This method is inexpensive.
- Non-corrosive to instruments.
- Non-hazardous to staff.
- Compatible with current equipment and procedures used in hospital sterilisation units.

## **5.7 Development and use of contamination /decontamination assays on surfaces**

### **5.7.1 Studies of prion protein on stainless steel surfaces**

Professor Keevil's group have looked at the effect of drying time, sonication, ambient temperature and pre-washing on ME7 contamination of surfaces and assessed this by SYPRO Ruby with EDIC/EF (described in chapter 2).

The experiments used prion-infected brain material from female C57BL/6J mice, which had been injected with 1 ml of 10% (w/v) ME7-infected brain homogenate into the dorsal hippocampal region of the brain. All animals were killed at 19-21 weeks post-inoculation or equivalent age for the negative group. One ml samples of the brain homogenate were placed on 'pristine' stainless steel tokens.



### **5.7.2 Effect of drying time**

The ability of commercially available SSD cleaning solutions to remove contamination (ME7-infected and normal brain homogenates) from metal tokens, and the effect of contaminant drying times on this ability was tested. The soiling was 'dried-on' for set time periods: 15 minutes, 4 hours and 17 hours and the tokens (316L stainless steel – approx 25mm x 5mm) cleaned under simulated washer/disinfector conditions. In general, it appeared that there was some relationship between drying time and the residual proteinaceous contamination remaining after 30 minutes of cleaning, with those tokens that were left to dry for longer being more difficult to clean.

This would indicate that a reduction in the delay between surgical instrument application and their reprocessing may be highly beneficial in improving the efficacy of SSD cleaning.

#### **Key findings**

- There is a positive relationship between drying time and the residual proteinaceous contamination.
- The longer soiling is allowed to dry, the more difficult it is to clean.

### **5.7.3 Effect of pre-washing with water**

This experiment was aimed to assess the possible benefits of a pre-wash step in the reprocessing of instruments and to give an indication of the time frame before pre-washing (initially in filtered distilled water) becomes ineffective. Infected brain homogenate (ME7 prion) of known protein concentration was applied to stainless steel tokens (25mm x 5mm) for set contact times; 0, 2, 5, 10, 15 and 30 minutes at room temperature. The work also looked at two different stainless steel types, 316L and 430. The tokens were then placed into filtered distilled water, stained and visualised to assess the degree of residual protein remaining. Results indicated that the adsorption curves possessed some similar characteristics; initially after only short periods of time (0 - 5 minutes) there was little residual protein 'attached' to the tokens' surface, however, between 5 - 15 minutes of contact time, a sharp rise in the degree of protein attachment occurs, which then subsequently plateaus (15-30 minutes).

It appears that pre-washing should be performed as soon as possible (preferably between 0 - 10 minutes) after instrument contamination in order to reduce the amount of residual soiling that requires removal/inactivation through the SSD.

#### **Key findings**

- Prewashing after a contact time with soiling of <10 mins at room temperature significantly reduces the amount protein contamination on stainless steel.

#### **5.7.4 Effect of sonication**

Experiments were conducted to investigate the effect of sonication on the removal of protein contamination on 316L suture wires. The effect of sonication (1 min) in a cleaning solution on its own, as well as sonication and a subsequent cleaning chemistry washing step under simulated WD conditions was examined. The results were then combined and compared with other wire experiments that had applied only the cleaning chemistries under the same simulated WD conditions.

When compared with the washing-only experiments, the findings indicated that for all of the cleaning chemistries, a reduction in the degree of contamination had occurred after the application of sonication only. However a more significant reduction occurred when these wires were exposed to subsequent simulated WD cleaning: with 50% (2/4) of the cleaning solutions tested displaying >1 log reduction in the residual contamination between just washing and sonication-washing.

Although the duration of sonication needs further investigation, it appears that sonication prior to cleaning may be highly beneficial in aiding the reduction of residual soil levels on surgical instruments.

#### **Key finding**

- Sonication prior to cleaning may be highly beneficial in aiding the reduction of protein contamination on stainless steel instruments.

#### **5.7.5 Effect of ambient temperature**

The protein adsorption experiments were repeated at set temperatures: (22°C, 30°C, 37°C). A clear shift of the inflexion point from approximately 10 minutes for 22°C and only 5 minutes for 30°C was obtained. The gradient of the increase in protein adsorption with respect to time was less at 22°C than 30°C. It was also observed that the difference between the adsorption curves at 30°C and 37°C was relatively small.

The findings indicate that the ambient temperature has a significant effect on the rate of protein adsorption, with lower temperatures giving a longer low adsorption period. The regulation of surgical instrument ambient temperature should be considered postoperatively.

#### **Key findings**

- Ambient temperature has a significant effect on the rate of protein adsorption.
- Lower temperatures (22°C) gave a longer low adsorption period than at 30°C or 37°C.

### **5.7.6 Effect of pre-soaking**

Subsequent experiments compared the effect on protein adsorption of pre-soaking for 5 minutes using five commercially available washing solutions after various drying times and at temperatures of 22°C and 30°C.

The pre-soak solutions were deionised water (control), Klenzyme, Endozyme AW, Enzol and Liquid 52. All the pre-soak solutions reduced the subsequent protein remaining on the surgical steel surface. The extent and efficacy of this removal varied considerably.

The maximum amount of adsorbed protein remaining after exposure to the pre-wash occurred more rapidly at the higher temperature. However the pre-soaks were able to delay this time with the maximum adsorption being from <8 minutes at 30°C to >30 minutes, compared with the deionized water pre-soak control. A clear pattern was seen with all the washing solutions, with an initial period of low adsorption followed by a rapid rise in residual protein and a subsequent plateau in adsorption levels.

Enzol appeared to perform this task best, lengthening the time taken for adsorption and removing 96% of proteinaceous contamination. However this still left around 4 ng/mm<sup>2</sup> of protein on the stainless steel surface. Considering the average prion molecule to be approx 30 kDa in mass, and one infectious unit to be 105 PrP<sup>Sc</sup> molecules, then around 6-105 IU/mm<sup>2</sup> could still be present before the WD cycle. It would therefore be preferable to develop improved pre-soak solutions to remove proteinaceous contamination before reprocessing.<sup>29</sup>

### **Key findings**

- All pre-soak solutions reduced protein contamination of instruments.
- Enzol most effective pre-soak solution.
- Pre-soaking alters the kinetics of adsorption, lengthening the time taken for adsorption and reducing the fixed protein concentration remaining after treatment.

### **5.7.7 Effect of sonication and cleaning chemistries**

The effect of sonication for set times (between 0 and 10 minutes) with nine different cleaning chemistries was investigated. The combination of sonication and cleaning chemistry significantly reduced the degree of protein contamination compared to water, which had little effect. During this cleaning process there was initially a rapid fall in proteinaceous load followed by a levelling off. After 5 minutes of sonication between 1 and 3 logs of contamination was removed. This is compared to only a 0.5 log reduction by the application of the chemistries alone.

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<sup>29</sup> Lipscomb IP, Pinchin HE, Collin R & Keevil CW. (2007b). Effect of drying time, ambient temperature and pre-soaks on prion-infected tissue contamination levels on surgical stainless steel: concerns over prolonged transportation of instruments from theatre to central sterile service departments. *J Hosp Infect*, **65**: 72-77.

In conclusion it appears that the results are encouraging with some cleaners nearing the sensitivity limit of the protein detection assay (175 pg/mm<sup>2</sup>). However there is still an indication that a robust, highly adhered sub-population of protein soiling can remain even after 10 minutes of sonication.

### ***Key findings***

- After 5 minutes of sonication in combination with different cleaning chemistries reduced protein contamination by between 1 and 3 logs.
- Highly adhered sub-population of protein soiling can remain even after 10 minutes of sonication.

### ***5.7.8 SYPRO Ruby/Thioflavine staining of PrP<sup>Sc</sup>***

The dual staining method using SYPRO Ruby/Thioflavine (detection limit <100fg PrP<sup>Sc</sup>; approx 2 attomoles) described in Chapter 2 was used to examine the effect of cleaning chemistries on PrP<sup>Sc</sup> contamination. Under sub-optimal cleaning conditions with an enzymatic cleaner, the degree of PrP<sup>Sc</sup> soiling remained constant even though the total amount of protein contamination was reduced. These findings would indicate that the PrP<sup>Sc</sup> appears more tightly bound to the surface, and/or that non-PrP<sup>Sc</sup> proteins are removed preferentially.

## ***5.8 Decontamination of luminal endoscopes***

The Southampton group have investigated the adhesion of proteinaceous material to the channels of luminal endoscopes, and determined the efficacy of current methods of decontamination.

### ***5.8.1 Scoping study for endoscopes***

Endoscope manufacturers/retailers and also SSDs where endoscopes are processed were visited to determine the particular problems of endoscope design, choice of construction materials, clinical use and post-operative cleaning requirements. Subsequently, an assessment was made of the suitability of a sensitive contamination detection technique developed by the group at Southampton - EDIC/EF microscopy, for use on intact endoscopes and their construction materials. The work showed that the EDIC/EF microscope detection procedure was very sensitive and reliable on a range of metallic and plastic surfaces, such as those found in endoscopes, with 100% of blinded observers being able to detect proteinaceous contamination down to 40 pg/mm<sup>2</sup>.

### ***5.8.2 Effect of temperature and contact time***

Studies to assess the physico-chemical characteristics of endoscope channel contamination, using the Medisafe test soil at various dilutions, contact time and temperatures, are still ongoing. Initial results show that the temperature of the soil (20°C or 37°C) and time of contact (up to 10 minutes) had little effect

on initial deposition in any of the channels studied. Deposition appeared to occur within the first seconds of contact, and led to a concentration-related protein deposition within the lumen (test soil was applied from 1 to 100% in pure water). The principal manufacturer of endoscopes sold in the UK, Olympus, would not communicate any technical information regarding the structure and composition of the various channels studied, other than these are made of PTFE, though at least two types of polymeric structures have clearly been identified under EDIC/EF microscopy. Although initial data indicated that large biopsy channels (O = opaque material) bind more material than the smaller air/water channels (T = transparent material), the cleaning studies which have been analysed so far revealed that more residual contamination remains on T-type channels after the simulated SSD pre-wash.

### ***5.8.3 Effect of soil drying, precleaning and brushing***

The different scenarios to which used endoscopes might be subjected were mimicked. Drying the soil (for up to 1 hour at 37°C according to the manufacturer's instructions) resulted in less effective pre-cleaning (with no brushing involved). Immediate soaking for up to 20 minutes in an enzymatic solution at the recommended temperature led to better removal than just maintaining the lumen wet with pure water. All concentrations of soil left small remaining particles in the tubing, following washing with various enzymatic solutions and rinsing with pure water. It was reported that there seemed to be more of these remaining small particles in the "T-type" channels (air/water and new biopsy channels) than in the "O-type" biopsy channels, if no brushing was performed. Brushing improved the removal of large deposits, and improved the removal of small particles in the "T-type" biopsy channels. There was eventually less remaining contamination in the "T-type" channels than in the "O-type" channel following "ideal" SSD initial wash protocol (channel maintained wet in enzymatic solution, brushing performed with fresh enzymatic solution, rinsing). This might be due to an aggregation effect.

The pre-cleaning step of endoscope reprocessing, before endoscopes are placed in a specific WD is critical, without it the remaining contamination is less likely to be removed during the subsequent processes and could potentially cross-contaminate the washer disinfectant itself.

The other main aspect of the project to evaluate current decontamination practice, and various protocols to investigate the different parameters of the cleaning step (immersion time, temperature, brushing, etc.) are still under investigation. Data collected so far indicates that Endozyme AW Plus (Ruhof), an enzymatic cleaner currently used in SSDs as a pre-wash prior to placing the endoscopes in WDs, has a limited efficiency in removing proteinaceous material when compared with other cleaning solutions; this is especially true for the air/water "T-type" channels.

In summary, data confirm that prevention of endoscope drying is the first important condition for endoscope decontamination (ideally with an enzymatic solution at correct temperature, since initial experiments indicated that water alone contributed in some cases to increased binding of soil prior to washing). Brushing was critical, since it appeared to facilitate the removal of otherwise tenacious protein deposits inside the "T-type" biopsy channels, which are the

new standard. However, none of the cleaning solutions tested led to total removal of protein contamination. An evaluation will be made of the recently commercialised cleaner, Prionzyme, to determine if it meets the manufacturer's claims

Further work will look at the potential effect of accumulation following repetitive use, as well as the effect of long-time wearing of the biopsy channel with forceps. The present work will finish shortly and future studies should consider detailed examination of endoscopes obtained from clinical use using the procedure developed here.

### ***Key findings***

- Endoscopes should be kept wet after use.
- Pre-washing step important.
- Pre-washing with enzymatic cleaner for 20 mins is more effective than water.
- Brushing important – removes deposits in both channels.
- None of enzymatic cleaners led to total removal of protein contamination.

### ***The Southampton group's collaborations with other researchers and industrial partners***

- Prof J. Grassi, CEA Saclay, France.
- Prof E. Abel, University of Dundee.
- Dr A. Byrne, University of Ulster.
- Prof C. Lowe, University of Cambridge.
- Dr L. Bountiff, Scottish Agricultural Research Institute.
- Dr C. Whitworth, University of Liverpool.
- Microgen Bioproducts Ltd, Camberley.
- Best Scientific, Swindon – microscope patent.
- Keymed, Southend.

## **6 GAS PLASMAS AND OTHER PHYSICO-CHEMICAL TECHNOLOGIES FOR DECONTAMINATION**

### **6.1 Low pressure radio-frequency gas plasma**

The MIDAS group set up at Edinburgh University and led by Professor Bob Baxter have developed and validated the effectiveness of low pressure RF gas-plasma technology as a means of removing protein contamination on surgical instruments.

#### **6.1.1 Development of RF gas-plasma**

A RF gas-plasma is formed when gases are subjected to an electric field. The RF field accelerates electrons which collide with gas molecules. The ionisation of gas molecules through inelastic collisions generate further electrons initiating a cascade. As in most electrical discharges, the gas volume is quite luminous (the glow discharge region). A sheath region of negative charge surrounds surfaces in contact with the plasma. The difference in electrical potential between the two regions causes a flux of positive ions to accelerate across the sheath and strike the substrate at high kinetic energy. The first effect of a gas-plasma on a substrate is one of chemical-etching by excited atoms and this has been likened to 'molecular sandblasting.' The molecules of the target also react with the ions and radicals formed in the plasma and result in radical formation in the target molecule and eventually to molecular cracking by mechanisms similar to those involved in the thermal cracking of petroleum. The aggressiveness depends on the gas mixture, the pressure and the temperature.

#### **Mechanisms of plasma action**

Studies have shown that hydration of the protein prior to treatment is necessary to achieve effective removal. The reasons for this are still unclear but it is thought, based on results from optical emission spectroscopy, that hydroxyl radicals are formed in the hydrated protein matrix and that these are, at least in part, responsible for acceleration of protein oxidation. Under typical experimental conditions using a Plasma-Etch PE 200 Instrument [Temperature 20°C; Ar:O<sub>2</sub> (1:2) mixture (at 0.280 Torr) subjected to RF excitation (13.5MHz) at a power density of ~6mW.cm<sup>-3</sup> for 1 hour] oxidation of protein samples occurs, and only CO<sub>2</sub>, H<sub>2</sub>O and NO<sub>2</sub> can be detected by in-line mass spectrometry of the effluent gas.

#### **6.1.2 Removal of protein by RF gas plasma**

To evaluate the effectiveness of the procedure, experiments were carried out with fluorescently labelled proteins where the protein residue could be accurately measured using a spectrofluorimeter with modified fibre optics. Under the above conditions, stainless steel surfaces (316) were cleaned to below the sensitivity of this assay (10 femtomoles/mm<sup>2</sup>, i.e. ca 0.5 ng/mm<sup>2</sup>). This technology has now been patented.

### **6.1.3 Removal of TSE infectivity**

Studies have shown that TSE infectivity can be removed from stainless steel by this treatment. In these experiments it was shown that while stainless steel spheres (316), coated with a brain homogenate from 263K scrapie infected mice and implanted intraperitoneally in hamsters, could transmit the disease, the transmission of infectivity was effectively eliminated by Ar:O<sub>2</sub> RF gas-plasma treatment of the spheres prior to implant. An important finding in this work is that, as with the pure protein studies, pre-soaking the tissue deposits in water immediately prior to RF-plasma treatment greatly improves the efficiency of the cleaning process.<sup>30</sup>

### **6.1.4 Studies on surgical instruments**

The efficacy of RF gas-plasma cleaning on a large number of different types of surgical instruments after conventional SSD cleaning and autoclaving, from different NHS Trust SSDs were used. Both decommissioned and 'in use' instruments were examined, these included (disposable bone files, Allis tissue forceps, and Gilles skin hooks). Instruments were examined by SEM and areas of contamination characterised using EDX prior to RF gas-plasma treatment and then re-examined after treatment. In certain cases where the instruments were not returned for re-use, examination of deposits was also carried out by surface protein labelling using fluorescent probes (see Chapter 2). In most cases decontamination was achieved to below the levels of SEM visualisation. There are two exceptions – where protein deposits are associated with large amounts of bone debris (e.g. orthopaedic saw blades) or entrained in salt crystals. Two RF gas-plasma cycles with an intermediate water wash were required to clean the surfaces.<sup>31,32</sup>

Typically, the instruments required one hour of plasma treatment and no damage to the surface of surgical grade stainless steel or to samples of silver, titanium and plastic was apparent after plasma treatment.

### **6.1.5 Studies on neurosurgical instruments**

In a collaborative study with the Department of Neurosurgery at the Edinburgh Western General Hospital, the retention of tissue contamination on surgical instruments from a single neurosurgery instrument tray, which had been through multiple surgical and reprocessing procedures was examined.

The control group of instruments were subjected to normal hospital SSD cleaning and sterilisation by autoclaving. A second group was subjected to RF

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<sup>30</sup> Baxter HC & Baxter RL. (2005). RF Gas-Plasma Treatment for Decontamination of Surgical Equipment and the Prevention of Transmissible Spongiform Encephalopathy Infection: *Managing Infection Control*, **5**: 1-4.

<sup>31</sup> Baxter HC, Campbell GA, Richardson PC, Jones AC, Whittle IR, Casey M, Whittaker AG & Baxter RL (2006). Surgical Instrument Decontamination: efficacy of introducing an argon:oxygen RF gas-plasma cleaning step as part of the cleaning cycle for stainless steel instruments. *IEEE Trans. Plasma Sci.*, **34**:1337-1344.

<sup>32</sup> Whittaker AG, Graham EM, Baxter RL, Jones AC, Richardson PR, Meek G, Campbell GA, Aitken A & Baxter HC. (2004). Plasma cleaning of dental instruments. *J Hosp Inf*, **56**(1):37-41.



gas-plasma treatment after the normal cleaning and sterilisation. In all cases the instruments were examined by scanning electron microscopy (SEM) directly after SSD reprocessing and sterilisation and residual contamination was subjected to elemental analysis by EDX. The second group was re-examined after RF gas-plasma treatment. The tray of instruments was then returned to the SSD, reprocessed, autoclaved and returned to surgical use. This resulted in one additional cleaning procedure being inserted into each normal cycle and the process was carried out over four complete use/cleaning cycles. A common pattern was evident over the control group of instruments examined. In some areas the overall profile of contamination was random and changed with each cycle. However, in other areas, the same residual contamination was evident over multiple cycles.

### ***Key findings***

- Clear evidence of some residues building up with repeated use/cleaning cycles.
- RF gas-plasma treatment was effective in removing all organic residues.

### ***6.1.6 Studies on endodontics drills***

In a separate study the group demonstrated the effectiveness of RF gas-plasma treatment for removal of tissue contamination from endodontal drills. Two plasma cleaning cycles were needed and results were confirmed by elemental analysis.<sup>33</sup>

### ***Key technical features***

- Plasmas can remove all surface protein, yet cause little surface damage.
- Plasmas can remove TSE infectivity.
- Plasmas can penetrate lumens and cavities.
- Large-scale equipment is already made for other purposes and significant experience has been obtained from their use.
- Samples with moderate levels of contamination require approximately one hour of treatment.

### ***Development of technology***

The group have recently entered into collaboration with Plasma Etch Inc., Carson City, (USA) who have constructed a prototype low pressure RF gas-plasma instrument (the PE BT-1) to the group's specifications. This is suitable for development as a machine for medical instrument decontamination in a

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<sup>33</sup> Whittaker AG, Graham EM, Baxter RL, Jones AC, Richardson PR, Meek G, Campbell GA, Aitken A & Baxter HC. (2004). Plasma cleaning of dental instruments. *J Hosp Inf*, **56**(1):37-41.

SSD environment and for dental instruments. Further work is underway to establish the best conditions and protocols for this instrument in routine decontamination.

### ***Further work***

Further work is planned on;

- Bioassays using more infectious materials and modes of infection. i.e. assays using 301V in mice are currently being planned.
- Validation of improved protocols for gas-plasma treatments of surgical instruments with different machine configurations.
- Development of the in-house surface fluorescence assay method as a general method for accurate measurement of the effectiveness of protein removal by different cleaning procedures. This is a collaboration with NHS Scotland.

In the long term, an area which urgently requires fundamental study is the nature and mechanism of protein adherence to surfaces. The group has various plans for an experimental programme to investigate these but as yet has not identified any suitable source of funding.

### ***The MIDAS group's collaborations with other researchers and industrial partners***

- Prof E. Abel, University of Dundee. Characterisation of new surface coatings.
- Prof M. Kong, University of Loughborough. Analysis of the effectiveness of atmospheric gas-plasma treatments.
- Dr J. Walker, Porton Down. Comparative analysis of solution cleaning methods.
- Plasma-Etch Inc., Carson City, Nevada. Development of vacuum gas plasma instrumentation.
- Dr S. Bozzini, Politecnico di Milano. Analysis of surface adhesion on PEG/TiO<sub>2</sub> surfaces.
- Mr D. Hill, Scottish NHS. Development of new testing methods for HSU systems.

## ***6.2 Cold atmospheric pressure plasmas***

The bioelectrical engineering (e-Bio) group at Loughborough University led by Professor Michael Kong has conducted a study on ambient pressure removal of infective protein residues from medical instruments, in collaboration with Professor Bob Baxter's group at Edinburgh University.

The original objectives were to:

- Develop novel low-temperature atmospheric plasmas that produce, in a well-controlled manner, very high fluxes of plasma agent that can be applied flexibly to protein destruction.
- Develop 'atmospheric plasma cleaning' methods for protein removal from instruments with controlled damage to the instrument.
- Verify the operational safety of the methodology and compare to the RF gas plasma method being assessed by Edinburgh University.

### **6.2.1 Development of CAP systems**

When the project started there were no credible manufacturers for low-temperature atmospheric plasma sources at an operating temperature below 70°C (to allow for plastic cleaning). There are now a very few, but at the time that the project started there was no option of purchasing a CE-marked cold atmospheric plasma (CAP) system. A major component of the project was to develop a CAP system.

To provide as wide a parameter range as possible for effective decontamination, a range of cold atmospheric plasmas (CAP) have been designed, constructed and tested. These CAP systems have been improved by; (a) changing the excitation frequency from 1kHz to 30MHz (> 4 orders of magnitude); (2) changing the background gas with different combinations of helium, argon, air, and oxygen; (3) using different treatment modes (remote, *in-situ*).

The CAP systems developed from the project can produce plasma covering lengths from 20 µm to one metre, making it possible to deal with instruments of a wide range of dimensions, and devices including endoscopes. Through physical techniques (e.g. optical emission spectroscopy, plasma physics simulation, electron energy dispersion X-ray), these plasma systems have been improved considerably over the course of the project and can deliver very high fluxes of agents, each capable of degrading protein in principle. The latter include charged particles (e.g. ions and electrons), metastables, excited and ground states oxygen atoms, UV photons, electric fields and shock waves.

The most favourable system developed is a CAP torch generated at 20–30 kHz and operated with an optimised helium-oxygen gas flow. The CAP torch produces a thermally gentle plume with abundance of chemically reactive plasma species consisting mainly of helium and oxygen.<sup>34,35,36</sup> Collaboration with Edinburgh University provided a surface protein detection method using

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<sup>34</sup> Deng XT, Shi J, Shama G & Kong MG. (2005). Effects of microbial loading and sporulation temperature on atmospheric plasma inactivation of *Bacillus subtilis* spore. *Applied Physics Letters*, **87**: 153901.

<sup>35</sup> Yu H, Perni S, Shi JJ, Wang DZ, Kong MG & Shama G. (2006). Effects of cell surface loading and phase of growth in cold atmospheric plasma inactivation of *Escherichia coli* K12", *J Appl Microbiol*, **101** (6): 1323 – 1330.

<sup>36</sup> Vleugels M, Shama G, Deng XT, Greenacre E, Brocklehurst T & Kong MG. (2005). Atmospheric plasma inactivation of biofilm forming bacteria for food safety control. *IEEE Transactions on Plasma Science*, **33**(2): 824-828.

laser-induced fluorescence; and this technique was used to evaluate the protein-removal capability of the CAP torch for (a) stainless steel disks with FITC-labelled BSA deposit and (b) real surgical instruments (e.g. forceps).

### **6.2.2 Degradation of BSA by CAP**

Using the laser-induced fluorescence technique for surface protein measurement and the CAP torch, a maximum BSA reduction of 4.5 logs was achieved for a total treatment time of 5 minutes: this corresponds to a minimum surface protein of 0.36 femtomole/mm<sup>2</sup>. Electrophoresis was used to demonstrate that BSA removal is most likely to be through protein degradation and that its typically biphasic reduction kinetics is influenced largely by the thickness profile of the surface protein.<sup>37,38</sup> To understand the identity of plasma agents responsible for the observed protein degradation, optical emission spectroscopy was used to correlate the production of key plasma species with the extent of protein removal under different plasma conditions (including oxygen admixture into the background helium flow). It has been established that oxygen atoms play a very important role, whereas UV contribution is minor.<sup>39</sup> Plasma simulation at Loughborough and elsewhere suggests that ions are less important because their energy is very low at atmospheric pressure.<sup>40,41</sup> The dominant role of oxygen atoms is desirable because they can access crevices and micro-scale spaces through gas diffusion: this is preferable to UV and ions that tend to travel in a single direction.

### **6.2.3 Degradation of contamination on surgical instruments by CAP**

Though not planned in the original proposal, the work has moved beyond the model protein of BSA as a result of recognising that BSA data are insufficient for extrapolation to predict the CAPs ability to act against prion. The cost of appropriate bioassay experiments was not budgeted in the current project, and therefore an alternative method was used: autoclaved surgical forceps that were ready to be returned to hospitals for re-use. A statistical analysis of bio-burdens was conducted using SEM and EDX, and surface proteins at 20 pg/mm<sup>2</sup> were observed. Although of unknown biological identity, these proteins were deposited on forceps following surgery and so presented a real-world test to the CAP cleaning system. By adding a subsequent sanitation step, the same CAP torch (as used in the BSA work) was found to effectively remove the proteins. This result suggests that the CAP technology is effective

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<sup>37</sup> Deng XT, Shi JJ & Kong MG. (2007). Protein inactivation by atmospheric pressure glow discharges: capability and mechanisms. *J Applied Physics*, **101**: 074701.

<sup>38</sup> Deng XT, Shi JJ, Chen HL & Kong MG. (2007). Protein destruction by atmospheric pressure low pressure glow discharges: capability and mechanisms. *Applied Physics Letters*, **90**: 013903.

<sup>39</sup> Deng XT, Shi JJ & Kong MG. (2007). Physical mechanisms of inactivation of *Bacillus subtilis* spores using cold atmospheric plasmas. *IEEE Trans. Plasma Science*, **34**(4): 1310-1316

<sup>40</sup> Shi JJ & Kong MG. (2006). Evolution of discharge structure in capacitive radio-frequency atmospheric-pressure microplasmas. *Physical Review Letters*, **96**: 105009.

<sup>41</sup> Shi JJ & Kong MG. (2007). Electron kinetics in radio frequency atmospheric pressure microplasmas. *Physical Review Letters*, **99**: 075004

for removal of human proteins deposited on surgical instruments following surgery and is at a stage to be developed further, subject to a rigorous bioassay experimental study. It also represents the first evidence that CAP can degrade and denature both purified protein and human proteins.<sup>42</sup>

Both the BSA model and forceps study have demonstrated a high level of decontamination by CAP, suggesting that any possible remaining contamination is beyond current detection methods (e.g. SEM, EDX, and laser-induced fluorescence).

#### **6.2.4 Mechanisms of plasma inactivation**

As an added objective to the original project, a collaborative study with Birmingham University was undertaken to understand the mechanisms of plasma inactivation. Here *E. coli* mutants were used: not as contaminants but as sensors for plasma species. By combining the *E.coli* sensor with physical techniques (e.g. optical emission spectroscopy) it can be suggested that oxygen atoms are the most significant plasma agents for inactivation of both proteins and bacteria.<sup>43</sup> Understanding of the mechanisms of inactivation is important for eventual FDA approval as well as for future modification and scaling up of the CAP decon technology to maximise the production of oxygen atoms.

#### **6.2.5 Operational safety and comparison with low-pressure plasma decon**

Based on a helium-oxygen gas mixture, the CAP torch has a gas effluent made mainly of helium, oxygen and nitrogen species (nitrogen coming from the ambient air).

At their ground states, helium atoms and oxygen and nitrogen molecules are harmless to humans and the environment, as are excited helium molecules. Reactive species such as metastable oxygen molecules, excited and ground state oxygen atoms, ozone, and various nitrogen species are short-lived, with half-lives often lasting some tens of microseconds - but typically below a few milliseconds - after which they recombine to become ground-state oxygen and nitrogen molecules. Such short time-scales make it realistic to control their impact on substrate surfaces, human operators and the environment. Tests were performed to determine the extent of surface damage by CAP on stainless steels and PTFE. SEM images of the surface suggest very little morphological change after CAP treatment.

Mass-spectrometry experiments were performed in collaboration with Hiden Analytical. By-products in the effluent of plasma-treated protein experiments were not found to be harmful. While this needs further confirmation, the early signs support the current understanding that the CAP decontamination process is an environmentally friendly procedure.

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<sup>42</sup> Kong MG. (2008). Project 0070088. Interim Report .

<sup>43</sup> Perni S, Shama G, Hobman JL, Lund PA, Kershaw CJ, Hidalgo-Arroyo GA, Penn CW, Deng XT, Walsh JL & Kong MG. (2007). Probing bacteriacidal mechanisms induced by cold atmospheric plasmas with *Escherichia coli* mutants. *Applied Physics Letters*, (99): 073902.

Discussions with Professor Bob Baxter's group and with the low-temperature plasma community suggest that the effectiveness of low-pressure plasmas relies on a significant contribution from UV and energetic ions, in contrast to atmospheric pressure plasmas in which reactive neutral species such as oxygen atoms are dominant. Expertise in optical emission spectroscopy and other plasma diagnostics techniques at Loughborough University offers further collaborative opportunities with Professor Baxter's group.

#### **6.2.6 Engineering scaled-up system**

Work has already met, and exceeded, the original objectives. More needs to be done before the technology can be trialled and exploited for use in a healthcare setting. The group have voluntarily introduced an added objective – engineering a CAP-based cleaning system. This added objective aims to identify scientific performance criteria for a future scaled-up CAP-cleaning system. By combining optical emission spectroscopy and bacterial genomics, the group have established that the main biocidal plasma species is oxygen atoms. This is particularly important to know because future system engineering - particularly the up- or down-scaling of the CAP cleaning system - will be based on the preservation or enhancement of atomic oxygen production. Optimising the CAP system into a pilot unit that can be integrated into the current decontamination infrastructure will be an engineering challenge.

#### **Key findings**

- Established for the first time the effective capability of cold atmospheric plasmas in removing and denaturing BSA (by at least 4.5 logs after 5 minutes).
- Established the capability of cold atmospheric plasmas to effectively remove protein residues on 4 types of forceps that had been used in operation theatres and subsequently autoclaved.
- Identified ground-state oxygen atoms as the main inactivation agents with UV, OH radicals, nitrogen species and charged particles playing secondary roles.
- Developed a variety of cold atmospheric plasma sources from millimetre-scale plasma jet and jet arrays, through a meter long plasma curtain, to very long (up to a couple of meters) plasma generated inside very small channels of sub-millimetre diameters (for catheter applications).
- Little damage to the substrate surfaces (e.g. steel and plastics), and no toxic by-products.

#### **Further work**

Proof of concept to establish a cold atmospheric plasma system to degrade proteins has been achieved. These studies were intended to be based on BSA, as a model protein, deposited on stainless steel surfaces. Cleaning tests

of PrP-infected substrates were not planned, although they were recognised to be critically important for optimising the cleaning technology. Follow-on work to demonstrate the efficacy of CAP in decontaminating prion contaminated surfaces is necessary. This will also involve bioassay experimentation using appropriate prion models.

The project also offers new opportunities, for example the decontamination of endoscopes, as CAP can be generated within the small channel of a rigid or flexible endoscope. This application has already been demonstrated to the DH DECON group.

### ***Exploitation of research***

To support and/or supplement work done in this project, the group have already secured an EPSRC project for developing radio-frequency CAP systems using both experimental and computational methods; a DEFRA project for CAP decontamination of food-borne pathogens; and a grant from BBSRC for decontamination mechanisms.

### ***The Loughborough group's collaborations with other researchers and industrial partners***

To bring on board input from molecular biology and protein biology, e-Bio have been working with a number of university groups and industrial partners including:

- Prof B. Baxter, Edinburgh University.
- Dr G. Shama, Dept. of Chemical Engineering, Loughborough University.
- Prof C. Penn, School of Biological Sciences, University of Birmingham.
- Drs Jon Hobman and Cath Rees, Division of Food Sciences, University of Nottingham.
- MRC Toxicology Unit, University of Leicester.
- Hiden Analytical Ltd.

Discussions with other research suppliers within the DH DECON group and in USA and Canada are ongoing but collaboration is dependent on further research funding. These international networks and UK partnerships are valuable for Loughborough to advance and move the CAP Decon technology closer to the healthcare facility.

## **6.3 Ozone sterilisation**

This study is conducted by Dr Neil Raven's group at HPA, Porton Down. The objective is to evaluate a novel sterilisation protocol based on ozone as a method for the inactivation of TSE agents, specifically BSE/vCJD on surgical instruments and compare the technique with a standard steam sterilisation protocol. This study is being carried out in collaboration with the manufacturer

of the 125l ozone steriliser (TSO3 Inc, Quebec, Canada) who have already carried out studies on scrapie strains with the University of Montreal and Health Canada. These studies have demonstrated the potential of ozone to destroy prions as assessed by Western blot.

### **6.3.1 Bioassay evaluation of TSE inactivation by ozone**

Transmission study using VM mouse strain has been set up to investigate the effectiveness of the ozone steriliser on removing protein contamination (BSE 301V as the infectious agent) on wires that were then implanted into mice. The dynamic range of the wire implant model using the VM mouse strain was determined. TSE symptoms were demonstrated in animal groups across a 7-log dilution range. Transmission rates decreased to 10-15% beyond the  $10^{-4}$  dilution titration point thus reducing the range of the model to approximately between  $10^{-1}$  and  $10^{-4}$ . Histology will be used confirm these findings and will also confirm whether or not any of the non-presenting animals at the higher dilution groups were in fact disease free or asymptomatic at the defined endpoint of 550 days post implant.

Assessment of ozone and benchtop autoclave processes is well advanced with all bioassay groups set up. Autoclave studies (134°C, 18 min) are nearing completion with mean transmission rates of 186 days post implantation (sd. +/- 10 days) and 176 days (sd. +/- 24 days) for runs 1 and 2 respectively. A 100% attack rate was observed for run 1, with run 2 currently at 80%. The results from the ozone study are now emerging and initial results suggest that ozone is more effective than autoclaving. These results will be subjected to final histology assessment at the end of the study, which is forecasted to be end of 2008.

## **6.4 Electro-elution**

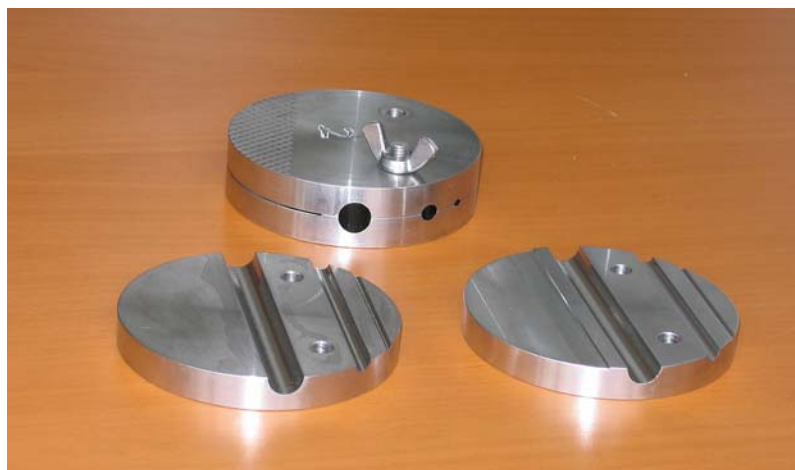
Dr Frank Prior has developed the process of electro-elution, a form of electrolytic cleaning to remove protein from surgical instruments. Testing of the process using TSE material was performed by Karen Fernie and Chris Plinston at the Neurophogenesis Unit in Edinburgh.

### **6.4.1 Development of Cleaning Test Disk**

Following identification of weaknesses in the use of visual soils (as recommended by HTM2030) for quality control (QC) tests of cleaning efficacy, a quantitative QC cleaning test was developed based on a cleaning test disk as a standard surface, and UV quantification of residual soil. The Cleaning Test Disk, is a “half-able” stainless steel disk which incorporates three different diameters of catheter grooves and a deep box joint (fig.2). A known amount of soil (e.g. blood) is pipetted into the grooves and box joint on one of the disk halves, and allowed to dry. The top half is then attached with a wing nut and the disk put through the cleaning process. At the end of the process, the top half is detached and any remaining soil on the lower half extracted with sodium carbonate solution. The amount of soil (blood) in the extract is



quantified using UV spectrophotometry using the major peak at 416nm for quantification of blood and the peak at 406nm for alcohol fixed blood. The percentage removal is calculated as the absorbance of the extract from the test disk divided by the absorbance of the extract from the control disk.



**Fig. 2. The Cleaning Test Disk**

Use of the cleaning disks has enabled the identification of factors that interfere with the cleaning process. The two major factors are alcohol and temperature fixation.

#### **6.4.2 Effect of alcohol, heat and disinfectants on blood removal**

A study was conducted to examine the effect of ethanol or isopropyl alcohol (IPA) on blood extraction from surgical steel instruments contaminated with human blood. Results showed that ethanol reduced the extraction of blood from the instruments by up to 60%. The binding effect was greatest at 75% ethanol in water, the concentration most commonly used in operating theatres. IPA caused a similar amount of binding but its effect was greatest undiluted.<sup>44</sup>

Studies examining the effect of heat have shown that exposure of blood contaminated instruments to 10 minutes at 50°C or 1 minute at 60°C resulted in fixation. Exposure of blood to alcohol or heat doubled the time required to elute blood from stainless steel surfaces.

#### **6.4.3 Effect of disinfectants on blood removal**

The initial prewash in most washer dryers consists of an initial cold prewash followed by a hot disinfectant rinse. The disinfectant rinse is generally at a high enough temperature to cause heat fixation. It is therefore important to ensure that the detergent will remove all the blood during the cold prewash. Operational studies conducted at Synergy Healthcare in Bellshill, Lanarkshire

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<sup>44</sup> Prior FGR, Fernie K, Renfrew A & Heneaghan G. (2004). Alcoholic fixation of blood to surgical instruments - a possible factor in the surgical transmission of CJD? *J Hosp Inf*, **58**: 78-80.

showed the cold prewash lasts for 7 minutes. Research was carried out to investigate if normal washer dryer disinfectants could remove blood and alcohol fixed blood within 7 minutes. Tests were performed to measure the percentage removal of dried porcine blood from external stainless steel surfaces (the cleaning disk with the lid off) and internal stainless steel surfaces. These tests were repeated using alcohol fixed blood on the test surfaces.

**Table 5. Blood removal after exposure to disinfectants**

Detergent	Blood removal (%)		Alcohol fixed blood removal (%)	
	5 mins	10 mins	5 mins	10 mins
Hamo 54 (internal)	71	73	37	-
Hamo 54 (external)	97	99	66	93
Hamo 100 (internal)	97	98	78	-
Hamo 100 (external)	97	99	98	99
Process pH plus	97	99	-	-
Endozyme	81	92	12	12

None of the above standard reagents produced complete removal of blood or alcohol fixed blood in 10 minutes. A more efficient method of removing blood protein from surgical instruments was required. Ideally such a method should;

- Remove blood and alcohol fixed blood from external and internal surfaces in less than 7 minutes.
- Completely remove prion protein in less than 7 minutes.
- Destroy prions in the process so that the eluent can be disposed of safely.
- Be simple and safe for operators to use.
- Be cheap enough to be used routinely in practice.

#### **6.4.4 Development of electro-elution**

Working on the hypothesis that prion may cold electroplate onto steel surfaces, processes were investigated that would reverse this galvanic fixation. Initial experiments using a 12 volt battery and sodium carbonate as the electrolyte demonstrated that blood could be rapidly cleaned from stainless steel surfaces by making the instrument the cathode of the electrolytic circuit. Further testing demonstrated that blood could be removed from external surfaces in less than two minutes and completely removed from internal surfaces in less than 10 minutes. The process was also effective in removing alcohol bound blood. The observation that the pink colour of the extract rapidly changes to colourless, and that the major UV peak shifted from

416nm to 406nm suggested that the protein was being denatured in the body of the electrolyte.

#### **6.4.5 Effect of electro-elution on removal of TSE infectivity**

In order to examine the effectiveness of “electro-elution” on the removal of TSE infectivity from stainless steel, Karen Fernie and Chris Plinston from the Edinburgh Neuropathology Unit undertook a pilot experiment to test the process. ME7 mouse brain homogenate was spread onto stainless steel disks which were then electro-eluted in aqueous carbonate solution buffered to pH7.5. A new process termed direct blotting was developed which utilises a process of passive transfer of proteins directly from the surface of the test disk to a proteophilic membrane for detection. The results of the study demonstrated definite positives on the control samples. However, no PrP<sup>Sc</sup> could be detected on the test disks after 1 minute of electro-elution at 0.2A in a 0.1% electro-elution buffer at pH 7.5. Examination of the carbonate buffer after electro-elution using Western blot analysis showed no detectable PrP<sup>Sc</sup> present, which suggests that the PrP<sup>Sc</sup> molecule has undergone some form of degradation or removal to the anode. Additional studies using bioassay techniques are required to confirm what is happening to the PrP<sup>Sc</sup> on the instruments themselves and in the body of the electrolyte and on the anode.<sup>45</sup>

#### **6.4.6 Effects on electro-elution on stainless steel surface characteristics**

The electro-elution process can result in metal burn however this is influenced by conditions during the electrolytic process. Experiments were performed to determine which factors affected surface characteristic of stainless steel. The current density required for successful electro-elution was 8-9 mA cm<sup>-2</sup> of stainless steel surface, whereas visible damage was observed at 70-100 mA cm<sup>-2</sup>. Thus, there is a good differential between the current required to electro-elute and the current which is likely to damage the instruments.

#### **Key findings**

- Blood could be removed from external steel surfaces in <2 mins and completely removed from internal surfaces in < 10 mins.
- Electro elution effective at removing TSE infectivity as no PrP<sup>Sc</sup> could be detected on the test disks after 1 min at 0.2A in a 0.1% electro-elution buffer at pH 7.5

#### **Further work and commercialisation**

These pilot experiments suggest that electro-elution may be a simple, effective and economical method of removing TSE infectious deposits from the surface of surgical stainless steel instruments.

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<sup>45</sup> Plinston C, Fernie K, Prior FGR & Smith R. (2007). Electro-elution, a novel method to remove transmissible spongiform encephalopathy – associated PrP<sup>Sc</sup> from stainless steel surgical instruments. *J Hosp Inf*, **66**: 52-58.

Experiments are presently under way to further characterise the variables employed in electro-elution, namely the current, voltage, and the strength of electrolyte as factors affecting the time required to remove blood and TSE infectivity. A research proposal to investigate the effectiveness of electro-elution in removing infectivity from stainless steel implants is presently being drawn up between Synergy and the Neuropathology Unit in Edinburgh.

The Cleaning disks (EP 1488757) and the process of electro-elution (EP1650329) have been patented. Confidentiality agreements have been signed with Getinge in Germany with the aim of developing an electro-elution chamber suitable for washer dryers. In addition a confidentiality agreement has been signed with Eschmann with regard to the development of a bench top electro-elution device suitable for dentists.

## **7 DEVELOPMENT OF BARRIERS AND SURFACE COATINGS**

### **7.1 Barriers for ophthalmic devices**

Professor Brian Tighe at Aston University leads a multidisciplinary team comprising Jonathan Kerr and John Flanagan (both clinicians); Howard Gee, Head of Regulatory affairs, First Water Limited; and Valerie Franklin. The team has worked to develop a sterile, universal barrier system for contact ophthalmic devices used in applanation tonometry.

The use of common ophthalmic contact devices results in a risk of cross-infection from prions (with the theoretical risk of CJD transmission); herpes simplex; adenovirus; HIV; and bacteria. The underlying problem is that tears contain blood-borne infective agents and present clinical standards for the sterilisation of ophthalmic contact devices (commonly wiping with an alcohol swab) are ineffective.

The project centred on the Goldman tonometer head, which provides the most accurate measurement of intraocular pressure (IOP). IOP informs diagnosis of glaucoma conditions and is used routinely in all hospital eye departments and by most optometrists in general practice. More than ten million IOP procedures each year are carried in the UK, so the potential for cross-infection is significant. Disposable prisms are commercially available that can reduce the risk of disease transmission, but these prisms are not considered convenient to use or of sufficient optical quality and are therefore not frequently used.

The aim of this project was to produce a sterile, universal barrier system for contact ophthalmic devices that would be inexpensive, effective, quick and easy to use, adaptable to all contact ophthalmic devices whilst maintaining their accuracy and effectiveness. The device takes the form of an optically transparent laminate barrier and consists of two thin layers: a barrier film with a sterile surface in contact with the eye; and a hydrogel, residue-free adhesive, with front and back surface covering that enables the film to be readily and reversibly attached to the tonometer head. The development of this barrier device involves several inter-related aspects, including device design, materials selection, device fabrication, and *in vitro* and clinical evaluation.<sup>46</sup>

#### **7.1.1 Materials selection**

Materials development and characterisation was carried out at Aston University, in close collaboration with Dr Howard Gee. Performance criteria for the barrier layer were identified and used to select forty candidate materials. The properties of these materials were measured with respect to suitability for fabrication, and with respect to the preliminary assessment of

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<sup>46</sup> Final Report 0070089. (2005). The development of a sterile, universal barrier system for use with contact ophthalmic devices.

operational performance. The properties measured included thickness or weight per unit area; optical transmittance; flexibility; barrier properties; and wettability or surface energy. Using this approach, polyurethane films were identified as the most promising material for the laminate barrier. The specification was:

- Thickness (<25  $\mu\text{m}$  uncoated - 250  $\mu\text{m}$  coated; weight <0.01g - 0.1g coated).
- Mechanical properties ( $E_{\text{mod}} < 50 \text{ Mpa}$ ;  $E_b > 250\%$ ).
- Optical transmission characteristics ( $T_{\text{wet}} > 40\%$ ).
- “Wettability” and surface energy ( $\gamma_p > 4 \text{ mN/m}$ ).

The required properties of the adhesive were found to be best fulfilled by a hydrogel. The critical aspects of performance were:

- Capable of being coated as a thin (< 100 $\mu\text{m}$ ) film.
- Showing good adhesive properties for PMMA tonometer tips.
- Leaving no residue.

An adhesive formulation was next designed that was based on these criteria and coated onto five selected candidate barrier materials to prepare the hydrogel laminates. Film thickness and adhesive strength were correlated in a preliminary assessment of the performance of the laminate.

### **7.1.2 Conversion of selected materials to prototype device**

To enable a useable device to be fabricated, barrier and adhesive needed to be:

- Assembled with readily removable front and back surface coverings.
- Reproducibly cut into device shape.
- Packaged and sterilised.

A series of iterations took place in which a number of potential barrier films, uncoated and in laminate form (i.e. coated with a hydrogel adhesive) were assessed to link the matrix of physical properties with the perceived clinical requirements and the validity of the initial design specification to be assessed. A polyurethane barrier material and ionic hydrogel adhesive combination was the preferred combination for prototype development and evaluation.

The sterile packaged device will be coated onto a readily peelable base layer, which would be removed before the adhesive layer is placed onto the tonometer head. Once attached in this way the backing strip will be removed to expose the barrier layer and prepare the device for use. In its final form the barrier device is a four layer composite.

The group assessed a number of commercially available materials to be used for the front and back liners, and a film produced by Smith and Nephew (EU31) was selected. A suitable manufacturing process has evolved, both for coating the hydrogel adhesive onto the barrier film and then for conversion of this laminate material into the required shape and format to permit its

application onto the tonometer head. This process has a high throughput with relatively low cost and has reached the production scale in First Water's coating lines.

### **7.1.3 Evaluation of prototype barrier**

Clinical Evaluation was carried out in two steps:

- Initial trials to establish the most appropriate design, coupled with ease of use and the ability to perform accurate tonometry. The first cycle of clinical evaluations started in October 2003, which provided information for material and design revision in time to start the second cycle of clinical evaluation in January 2004.
- A two stage trial, the first stage carried out in an optometric clinic to determine the difference between tonometer applanation results with and without a sterile barrier, and the second stage in a hospital ophthalmology department, to determine the differences in protein contamination following applanation tonometry with and without a sterile barrier.

The stage 2 Clinical Trial at Moorfields Eye Hospital recruited 69 patients and demonstrated that the applied adhesive film device gave an effective barrier to the proteinaceous components of the tear film and that the effectiveness and reproducibility of the measurement of the ocular pressure was not compromised by the presence of the device. Although this work was able to show the absence of protein species at the detection levels required, with hindsight a more appropriate technique would perhaps have been developed.

These trials have demonstrated that all aims of the proposal were successfully completed. A disposable, optically transparent, laminate barrier was developed both with a residue-free adhesive surface and with a sterile surface used for ocular assessment.

### **Cost estimates**

The cost target for the barrier system has always been 10p per piece at point of manufacture. The cost depends upon the complexity of design and scale of production because the raw material costs are only a small proportion of the cost of the packaged device at point of delivery. The proposed device will be significantly cheaper than the Tonosafe (approximately 55p) and cheaper and more accurate than the Tonoshield (approximately 25p) which are the existing disposable tonometer components. The ophthalmic instrument manufacturer Clement-Clarke has estimated that widespread use of Tonosafe devices would increase Moorfields Eye Hospital's annual budget by £350,000 and smaller hospital departments, such as the Brighton Eye Department, by £40,000 per annum.

### **Application to other contact optical devices**

In addition to contact tonometry there are a number of other ophthalmic instruments used routinely in hospital departments where the device (or part of the measuring device) comes into contact with the eye e.g. pachymeters

that are used for measuring corneal thickness. Consequently, there is scope for new work on development of suitable barrier devices for these instruments. The contact part of these instruments is quite different from the tonometer so different shapes and sizes (and hence potentially different polymer films) will be needed in order to provide an effective barrier interface.

It is believed that the principle of the disposable barrier is equally applicable to other devices, such as gonioscopes, A-scan lenses, and instruments used in vitrectomy procedures. There are no current disposable barriers for these devices and disposable lenses would be prohibitively expensive but because (unlike the tonometer) the optical surfaces are not flat, the materials and fabrication issues will be more complicated.

### ***Key technical features***

- Inexpensive and disposable.
- Impervious barrier to all infectious agents.
- No loss in optical quality.
- Applicable, in principle, to many contact optical devices.

### ***Future work***

Further work would modify the existing design for the adhesive film barrier device, without changing the component parts in their functionality, so that the device can be more and quickly applied to the tonometer head.

An issue identified by clinicians in the Clinical Trials was the time taken for the optometrist to place the device onto the head: this time should be as short as possible to encourage use in a busy practice or hospital department. Further work would need to look at modified designs with professional design input to develop more rapid application, such as through introduction of handling tabs and packaging devices, for example, in packs of ten on a single card. The design refinements need to be linked to trials with a number of clinicians in order to get a more general and wider view of its ease of use.

### ***Commercial exploitation***

The project team has obtained Health Technology Portal funding to refine the design and its manufacture, and to further evaluate candidate variations with a wide clinical base as a basis for widespread acceptance in the ophthalmic profession. A suitable marketing partner has been identified. A follow-up HTD grant was awarded but unfortunately this could not be taken up due to the untimely death of Dr Howard Gee (who was instrumental in the DH project as the link between First Water Ltd and the University) and the Universities' inability to agree the terms of the insurance indemnity as per the award.

Jonathan Kerr and John Flanagan are pursuing this project independently.



## **7.2 Diamond-Like Carbon coatings**

Professor Eric Abel and Dr Qi Zhao at Dundee University investigated the feasibility of altering the surface properties of surgical steel instruments and medical devices using new nanocoating technologies to minimise or eliminate adhesion to the coated surface by protein tissue, particularly prion-like proteins.

### **7.2.1 Material choice**

The material of choice was Diamond-Like Carbon (DLC), which has good biocompatibility, low friction and chemical inertness and excellent wear and scratch resistant properties. DLC is an excellent base coating because the amorphous nature of DLC opens the possibility of introducing small quantities of additional elements such as fluorine, silicon, nitrogen, boron or aluminium - or combinations of these elements - to alter the surface energy of the coating. Such a change in surface energy can repel material with particular surface energy characteristics while still maintaining the amorphous phase of the coating.

### **7.2.2 Theoretical modelling in choice of elements**

Theoretical and experimental studies at Dundee University, principally carried out by Dr Konstantin Borisenko, focussed on identification of a range of test surface coatings for evaluation. Two theoretical methods were used: the first using molecular-level computational chemistry to compute interaction energies between modified DLC surfaces and individual residue of protein/prion. The second method involved mesoscale-level Derjaguin, Landau, Verwey and Overbeek (DLVO) theory using supporting experimental data, to try to identify the optimal value of surface free energy at which the adhesion of a protein is minimal.

Using DLVO theory as the basis for modelling the interaction between a particle and a surface in the presence of a liquid, the four components affecting the surface energy are the Lifshitz-van der Waals interaction, the Lewis acid-base effect, the electrostatic double-layer and Brownian motion. The last component can be ignored. Tests carried out at Leeds University revealed that the electrostatic double-layer effect, measured by the zeta potential, was very small for all the surfaces, leaving the first two components as the main contributors to the surface properties and the main design variables for new coatings.

Using theoretical modelling at the atomic level, the adhesive properties of (111) diamond surface modified by introduction of an additional element - Si, N and F at a range of 2% to 8% was investigated. These three elements were chosen for their distinctly different electronic properties, so that a model could be established which identifies the predominant factors contributing to adhesion. Adhesion energies for surfaces doped with a series of representative peptides (hydrophobic Phe-Gly-Phe; amphiphilic Arg-Gly-Phe; and hydrophilic Arg-Gly-Arg) in a water environment were calculated using molecular dynamics simulations. The calculations suggested that substitution of atoms on diamond (111) surface will increase the polarity of the surface

and therefore make its interactions with water stronger. As a consequence, the adhesion energies of model peptides were found on average to reduce with an increasing amount of the substituting element for all elements (N, Si, and F). The strongest reduction was observed with an increasing amount of the N and F substituents. These observations have indicated that diamond surfaces doped with small amounts of Si, N or F should have better protein-repellent properties than those of pure diamond, and doping should increase the hydrophilic character of the material and therefore reduce protein adsorption.

### **7.2.3 Si and N doping and adhesion**

Modelling evidence indicates that doping with Si, N and F will reduce the hardness of the surfaces considerably. The group have investigated Si and N as a dopant. Based on the general finding from the molecular modelling study that elements with electronegativities that are substantially different from carbon should have hydrophilic properties and reduce protein adhesion, it was decided to include other example elements in the study. Al and B were selected. The coatings were applied by magnetron sputtering. Although all the doped coatings could give a lower level of residual protein after contamination and cleaning, there were inconsistencies in the results. A further inspection of the surfaces showed that many of the coatings were not attached properly, particularly after cleaning the surfaces. This was due to a manufacturing fault by a very well established coating company. The researchers arranged for another company to produce new coatings. To date only results from Si and N have been reported but data from other doping elements will be evaluated as soon as they are available. They were not ready for testing by the end of the project.

Three specified types of DLC coating containing different amounts of Si and N were prepared using a sputtering method. The actual content of the Si dopant was identified by XPS spectroscopy. The three surfaces contained either Si or N at 2%, 5% and 8%. Hardness testing showed about a 15% reduction for the Si-doped surfaces and 10% reduction for the N-doped surfaces, compared with pure DLC. All surfaces were at least five times harder than stainless steel. XPS revealed that a considerable amount of water adsorbs on the surface and an examination of the Si surface showed that the amount of adsorbed water seems to correlate with the amount of Si additive. These results - the increase in hydrophilic character with increasing Si concentration - appear to be in agreement with the theoretical molecular dynamics simulations of model diamond surfaces, which suggested an increase in hydrophilic character of the surfaces with increasing amount of Si additive in the films. The prepared coatings were submitted to the group's collaborators for evaluation of protein adsorption.

### **7.2.4 Si and N doped DLC and Brain Tissue adhesion**

DLVO theoretical modelling was performed using experimental surface energy data obtained for brain and liver tissue homogenates and the prepared Si- and N-doped DLC surfaces. In these calculations, equal electrostatic potentials of the surface and brain tissue proteins were assumed. With this

approximation, the ideal surface which repels the prion protein most strongly would possess a low van der Waals interaction component and a high value for the electron donor component of the surface energy. It was observed by contact angle measurements that doping by Si induced the type of trend in the change of surface energy parameters of the DLC films that were desirable in a coating.

Preliminary measurements of adhesion of brain tissue infected with TSE to the prepared Si-doped DLC surfaces have been performed by Professor Keevil's Group. These measurements have shown notable reduction in adsorbed and residual protein load after cleaning as compared to surgical stainless steel. The amount of adsorbed protein decreased with increasing amount of Si dopant in the DLC films.

### **7.2.5 Dual doping**

Work is underway to investigate dual doped coatings using combinations of Al, B and Si, which it is hoped will combine the best surface characteristics with appropriately hard surfaces suitable for surgical instruments. For example, Si has good non-stick properties but is less hard than DLC. Adding B will improve its hardness.

Modelling work shows that, for repelling prions, which have a positive charge, the best prion-repelling surface should have a similarly positive charge, low LW component of surface energy, a high electron donor component and a low electron acceptor component. This forms a basis for further work and for obtaining a better understanding of these factors as soon as the protein adhesion results can be correlated with the properties of the new surfaces. It is estimated that this information will be by the end of 2008.

Regarding modelling, more sophisticated molecular models will be required for the future in order to approach realistic models of the protein-surface interface and the surface energies at this interface. The information gained from the modelling process has been very useful to improve understanding of some of the factors, from which new information about the interaction between diamond surfaces and peptides which are hydrophobic and hydrophilic have been published (see Appendix 2).

### **Study with Southampton Group**

A small study was conducted to examine protein adsorption on 8 token types: 3 with each of the different Si and N-doping levels, 1 pure DLC and 1 stainless steel control. The tokens were inoculated with ME7-infected brain homogenate and allowed to dry onto the surface for 30 minutes. The tokens were then assessed for their initial protein load before being washed in a commercial cleaning solution for 5 minutes. The tokens were then reassessed.

The findings indicated that pure DLC coated tokens had adsorbed similar amounts of protein to stainless steel, but that after cleaning the residual load was typically about 50% for the pure DLC.

Increased Si and N concentrations allowed for better contamination removal using two different cleaning solutions for 5 minutes; it was noted that a proteinase-based cleaner (Enzol) was over two-fold more efficient than a lipase-based cleaner (Adyzime) resulting in much lower residual loads than that for stainless steel; as low as 6% for the 8% N-doped surface.

### ***Key findings***

- Demonstrated proof of concept using theoretical modelling and experimentally that doped DLC coatings can reduce adhesion of prion-contaminated brain tissue compared to stainless steel.
- Observed that for DLC doping in general it is advantageous to increase the hydrophilic character of the material and thereby reduce protein adsorption.

### ***Other uses of technology***

It has been speculated in the emerging field of nanomedicine, that a diamond coating may protect future nanorobotic devices operating inside the human body.<sup>47</sup>

### ***Collaborations***

Further work is planned, subject to funding, to test the contamination and cleaning of the doped DLC surfaces using a number of techniques with collaborators in London (Professor Perrett), Southampton (Professor Keevil) and Edinburgh (Dr Jones, Professor Baxter).

### ***Commercial exploitation***

- A patent has been granted for the DLC and other coating technologies.
- Private finance is being secured for the technology.
- Route to exploitation is being explored.
- Estimated cost of coating a typical instrument is about £1.50 but will vary depending on size.
- The life expectancy of the coating could be over 10 years with an appropriately high coating quality and coating thickness of about 2 micrometers. It will be much harder than stainless steel.
- Coating should not interfere with the functioning of the instrument although colour will be darker.
- Coatings are chemically very inert and do not react with chemicals used for decontamination.
- Coatings can work on a large scale as similar coatings for other industrial equipment have already been used in industry.

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<sup>47</sup> Borisenko KB, HJ Reavy, Q Zhao & EW Abel. Adsorption of protein residues on doped diamond (III) surfaces: theoretical molecular dynamics simulations. 17th European Conference on Diamond, Diamond- Like Materials, Carbon Nanotubes, and Nitrides, Estoril, Portugal, September 3-8, 2006.

### **7.3 Photocatalytic coatings**

Dr Tony Byrne and colleagues at the University of Ulster have begun work on the research and development of photocatalytic coatings as a potential alternative, cost-effective decontamination strategy for surgical devices. Photocatalysis has been reported to be effective for the killing of microorganisms (bacteria, fungi, and viruses) and cancer tumour cells.<sup>48</sup> The underlying theory is that instruments can be coated with a thin film of active titanium dioxide (TiO<sub>2</sub>), the most widely used photocatalyst, because it is inexpensive, chemically stable, photo-stable, photo-active, and non-toxic; and under UV irradiation it will actively degrade protein material adhered to the surface.

The objectives of the project were:

- To coat medical grade stainless steel coupons with a thin film of photocatalytic material.
- To contaminate the coated coupons with prion related material in a controlled manner.
- To irradiate the contaminated coupons with UV light under controlled conditions.
- To examine the surface of the coupons for evidence of decontamination following UV irradiation.
- To optimise the coating procedure to give hard, scratch resistant, self-cleaning films on the surface of stainless steel.

This was a proof of science project in the first instance, and the ultimate goal would be the production of surgical devices coated with 'self-decontaminating' photocatalytic films.

#### **7.3.1 Characterisation of films**

Photoactive TiO<sub>2</sub> thin films were prepared on borosilicate glass and stainless steel using two routes: sol gel and reactive DC magnetron sputtering. The films were characterised using X-ray photoelectron spectroscopy (XPS), X-Ray diffraction (XRD), scanning electron microscopy (SEM) with energy dispersive analysis of X-rays (EDX), and Raman spectroscopy. The films prepared by the sol gel method were prone to cracking where the film thickness was too great, resulting in stress due to contraction during drying. More uniform films could be prepared by the sol gel route using a serial process of multiple thin films. The films as deposited were amorphous and an elevated temperature treatment was required to obtain an anatase crystal form: this is desirable because it is the more photocatalytically active phase of

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<sup>48</sup> Blake DM *et al.*, (1999). Application of the photocatalytic chemistry of titanium dioxide to disinfection and the killing of cancer cells. *Separation & Purification Methods*, **28** (1); 1-50.

TiO<sub>2</sub>. The sputtered films were more uniform than the sol gel deposited films and did not require elevated heat treatment to give crystallinity.

### **7.3.2 Design of photoreactor**

A photoreactor was designed and constructed so that a number of samples could be irradiated under controlled conditions of light intensity and atmospheric humidity. It consisted of a Perspex cell which contained inlet and outlet gas ports, a quartz window and a stainless steel grid for sample support.

### **7.3.3 Determination of photocatalytic activity using model organic contaminants**

Experiments were undertaken (in collaboration with Edinburgh University) to determine if photocatalytic coatings (prepared via the sol gel route on glass) could decontaminate surfaces contaminated with BSA as a model protein contaminant. In these experiments, Fluorescein-isothiocyanate labelled (FITC)-BSA was used in order that the fluorescent signal could be correlated to the concentration of protein on the sample surface. A decrease in fluorescence intensity with increasing UVA exposure times for a range of initial FITC-BSA protein loadings suggested protein degradation. At higher loadings of protein, a lag period was observed and this was probably due to proteins screening the photocatalytic film from UV.

Further experiments used stearic acid as a model organic surface to determine the photocatalytic efficiency of TiO<sub>2</sub> films prepared on medical grade stainless steel using the sol gel route. These films were contaminated with a known amount of stearic acid by spin coating. The contaminated samples were irradiated for set time periods with a UVB source and the concentration of stearic acid determined by diffuse reflectance FTIR. Results showed a decrease in the relative stearic acid surface concentration as a function of irradiation time under different relative humidity. The optimal photocatalytic rate of decontamination of stearic acid was observed under conditions of 50% relative humidity. In the absence of TiO<sub>2</sub> (i.e. UV photolysis) there was negligible degradation of the stearic acid.<sup>49</sup>

### **7.3.4 Photocatalytic decontamination of rPrP<sup>C</sup>**

Experiments to investigate the photocatalytic decontamination of the prion protein rPrP<sup>C</sup> from MBH were conducted using TiO<sub>2</sub> films prepared on stainless steel via the sol gel route. The surface was contaminated with rPrP<sup>C</sup> and irradiated under a UVB source for up to 44 hours. Following treatment, the samples were exposed to a two step immunoassay which had been specifically developed for the detection of rPrP<sup>C</sup>. This involved a gold-labelled secondary antibody which could be detected using (scanning electron microscopy) SEM following gold enhancement. Results showed that the UV

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<sup>49</sup> Final report 0070090. (2006). Photocatalytic coatings for decontamination of prion contaminated surgical devices.

irradiated area had fewer gold particles on the surface than the non-irradiated area. The gold particles observed on the irradiated area appeared to be relatively larger than the average particle observed on the non-irradiated area, suggesting some formation of gold clusters at non-specific sites during the enhancement stage. In conclusion, while these experiments showed positive results in terms of fewer gold clusters after 44 hours of irradiation, the non-specific deposition of gold during the gold enhancement stage meant that results were not conclusive. Further experiments carried out with 50 nm gold label without gold enhancement were undertaken. However, EDX analysis was inconclusive for confirmation of gold label and presumptive gold spots were too dispersed to produce quantitative counts for comparison of controls and experiments.

Although the results of this study do not conclusively demonstrate the proof of science that photocatalytic films can effectively destroy surface-bound prion protein, there is evidence to warrant further investigation of this approach. Further work will be undertaken, beyond this project, which will investigate photocatalytic inactivation of infective prion material.

A number of drawbacks with the work were identified:

- Under low intensity UV conditions, photocatalysis can take a relatively long period for decontamination but this can be easily addressed by the use of high intensity UV sources.
- The process may not be applicable to recesses and cavities on devices that cannot be irradiated.
- Non specificity of gold enhancement step with labelled immunoassay and long analysis time by SEM.

Stability of the surface-cracking would impair functioning, however, TiO<sub>2</sub> coatings can be optimised for uniformity, hardness and wear resistance. Sputter deposited films do not suffer from cracking under the right conditions.

### ***Study with Southampton group***

New studies have begun to assess the potential of TiO<sub>2</sub> coatings on surfaces as a facilitator for decontamination. Stainless steel and PMMA tokens with and without TiO<sub>2</sub> coating were inoculated with ME7-infected brain homogenate and allowed to dry at room temperature for 30 minutes. The tokens were then exposed to UV light for up to 6 hours. Initial observations show a marked reduction in residual protein coverage on all surfaces after 6 hours, though protein removal from stainless steel surfaces appeared enhanced by the TiO<sub>2</sub> coating. Further work will be performed when possible to confirm these preliminary findings.

### ***Key findings***

- The photocatalytic process is slow but can be speeded up. This depends upon the incident light intensity and for surface decontamination which should not be mass transfer limited, the rate should be proportional to light intensity (up to maximum saturation intensity).

- Cost of coating a typical instrument is expected to be below £1 but will be affected by economy of scale. Sputter coating is used widely on an industrial scale for a range of surface coatings.
- Industrial scale coating of TiO<sub>2</sub> is already undertaken for self-cleaning glass by Pilkington (Activ glass). The methodology used for surgical instruments would be different.
- The coatings are expected to have a long life time. TiO<sub>2</sub> is a ceramic material and Mohs' Hardness of Abrasives gives a value of around 6 which is slightly less hard than steel - 8, diamond -10. However, the use of abrasives during cleaning should be discouraged as this would damage the coated surface.
- Decontamination technologies currently used in SSDs (washing with disinfectants, enzymes or autoclaving) should not impair the TiO<sub>2</sub> coating.
- How a high intensity UV disinfection stage can be incorporated into existing decontamination facilities would need consideration for progression of the technology.

### ***Further work***

Further work is required to optimise the coating technique and speed up the photocatalytic process. Collaborations are being pursued with other researchers within the DECON group.

### ***Commercial exploitation***

Based upon some of the work undertaken within this project, funding has been secured from Invest Northern Ireland Proof of Concept Programme for the commercial development of photocatalytic coatings for *in-situ* sterilisation of applanation tonometer heads. Efforts are ongoing to secure IPR for the technology. The Group have also secured funding from the R&D Office (NI) and the Health Research Board (ROI) for a collaborative study between Ulster and the Royal College of Surgeons in Ireland for the detection and removal of pathogenic biofilms from implantable medical devices. Further research funding has been secured via Science Foundation Ireland under their Northern Ireland supplement scheme to allow collaboration with Dr Tia Keyes at Dublin City University who has expertise in Raman spectroscopy and protein analysis on surfaces.



## **8 SUMMARY AND CONCLUSIONS**

The aim of the DECON research programme addresses the need to protect against the iatrogenic transmission of TSEs. The research projects supported can be divided into themes with considerable overlap. The main outcomes from the research are summarised.

### ***Development of assays to detect protein and prion contamination***

- Ninhydrin is inappropriate for the monitoring of protein contamination on solid surfaces.
- Some readily available stains (SYPRO Ruby, Thioflavine T) can reliably detect proteins on solid surfaces at the femtomolar level.
- Fluorescent probes can detect prions and other proteins at picomolar or attomolar concentrations on solid surfaces.
- Methods for detection by EDIC/EF microscopy and spectrofluorometric scanning have been developed and commercialisation of these is in progress.
- High sensitivity ELISA capable of detecting prions at picomolar level has been developed. Commercialisation of this is in progress.

### ***Assessment of current NHS procedures***

- Prions that cause human diseases, especially those arising from BSE, are resistant to heat inactivation and many other forms of physical and chemical disruption. Allowing proteinaceous deposits to dry out makes them difficult to remove and may increase the infectivity of adherent material.
- Surgical procedures result in tens of milligrams of tissue being deposited on instruments and when those instruments emerge from NHS SSDs they are frequently contaminated with significant amounts of protein.
- Dental equipment is contaminated with proteinaceous deposits.

### ***Detection of infectivity attached to implants***

- Infectivity bioassays have been developed with wires, spheres and direct injection.
- Assays require a large set of variables ranging from host genotype to treatment of inoculum. Assessing the effect of one variable e.g. strain of agent in these multivariate studies is very difficult.
- Efficiency of infection of TSEs is influenced by the physical nature of the homogenate i.e. wet or dry.

### ***Decontamination/ disinfection processes***

- Most common laboratory detergents are inefficient at cleaning steel surfaces.
- Many common commercial detergents, currently in use in NHS SSDs, can remove proteins from the surface of stainless steel instruments but these are not 100% efficient.
- Keeping instruments wet aids removal of contaminants.
- Pre-washing and ultrasonication can aid the removal of proteinaceous deposits.
- Pre-washing should be performed as soon as possible (preferably between 0-10 minutes) after instrument contamination, and that relative high ambient temperatures should be avoided in order to reduce the amount of residual soiling that requires removal/ inactivation through the SSD.
- Temperature, pH, humidity and type of detergent influence the degree of protein removal.
- Heat inactivation at high pH can result in complete inactivation of TSE agents without significant damage to high quality stainless steels.
- Heat based methods such as autoclaving do not completely inactivate TSE agents. It is hypothesized that the structures of TSE agents are stabilized during heat-inactivation procedures, rendering them much more refractory to inactivation.
- Proteolytic enzyme MC3, is now commercially available as Prionzyme™ and can remove virtually all bound infectivity.

### ***Development of decontamination technologies***

- Novel technologies, such as RF and CAP gas plasmas, could be used to clean instruments with crevices and lumens, and also those with sensitive components.
- RF gas plasma is effective at removing prion contamination and infectivity. Commercialisation of this technology is in progress.
- Ozone sterilisation is effective at removing protein contamination and more effective than autoclaving at inactivating TSE agent.
- Electro-elution effective at removing prion contamination.

### ***Development of barriers and coatings***

- An optically neutral barrier coating to prevent transmission by reusable ophthalmic instruments has been developed. Clinical trials confirmed clinically acceptable performance of the prototype device.
- Coating of surfaces with photocatalytic TiO<sub>2</sub> have shown that there is potential for instruments to be 'self cleaning'.
- Instruments coated with doped diamond-like carbon have demonstrated the feasibility of preventing initial attachment of proteins.

## **9 APPENDIX 1**

### ***Properties of PrP and potential impact on contamination of surgical instruments***

Dr Igor Bronstein has worked in collaboration with Dr Denise Dear, Prof. Iliia Baskakov, Prof. Igor Lednev and Dr Marina Kriajevska on a project which addresses first the development of appropriate cellular models to investigate the binding and associated toxicity of PrP protein and its amyloid derivatives to cell surface receptors and secondly the analysis of signalling pathways mediated by normal and pathogenic PrP species. In addition the researchers are planning to prepare a number of biochemical reagents (PrP protein with different glycosylation pattern and oligomerisation status) for their own use and for other researchers in the DECON group.

#### ***9.1.1 Development of Cellular Models***

Some of the original experiments to test the toxicity of the PrP protein were planned to be performed with animals, however due to unforeseen difficulties this was not possible. To achieve its' aim the group initiated the development of human and mouse cellular models. These models have used human and mouse neurospheres derived from human embryonic carcinomas (EC cells)<sup>50</sup> and mouse embryonal stem cells (ES cells), focusing on the toxicity and physiological activity of endogenous PrP material obtained from cellular homogenates with elevated level of PrP expression, from blood serum and from fibrillar recombinant protein. In addition, various oligomeric and fibrillar forms of recPrP protein were tested using the same cellular models. By exploring various tissue cell lines with elevated level of PrP protein and different patterns of glycosylation it was shown that several breast and prostate cancer cell lines of mesenchymal origin and with metastasis potential were characterized by extremely high level of PrP expression and unusual glycosylation pattern.<sup>51</sup> This observation raised a very important issue that surgical operations on metastatic tumours may stimulate the release of potentially toxic PrP species into the blood stream. Taking into account that surgical invasive procedures are able to activate a cascade of proteolytic enzymes including various cathepsins, further work analysed the effect of some proteases on the aggregation properties of recPrP. It was found that cathepsin S is able to specifically cleave recPrP protein and promotes the formation of protein nanoparticles with a very strong adhesion potential.<sup>52</sup>

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<sup>50</sup> Novitskaya V, Makarava N, Sylvester I, Bronstein IB & Baskakov IV. (2007). Amyloid fibrils of mammalian prion protein induce axonal degeneration in NTERA2-derived terminally differentiated neurons. *J Neurochem*, **102**(2): 398-407.

<sup>51</sup> Kriajevska M & Bronstein IB. PrP<sup>C</sup> protein as a new sensor of tumour microenvironment and a marker of metastasis. (Unpublished data).

<sup>52</sup> Polyakova O, Dear D, Stern I, Martin S, Hirst E, Bawumia S, Nash A, Dodson G, Bronstein I, Bayley PM. (2009). Proteolysis of prion protein by cathepsin S generates a soluble beta-structured intermediate oligomeric form, with potential implications for neurotoxic mechanisms. *Eur Biophys J*, **38**(2):209-218.

### **9.1.2 Structural conversion and aggregation of proteolytically digested PrP protein: potential impact on the protein contamination of surgical instruments.**

The factors causing protein aggregation and strong adhesion to metal and plastic surfaces are likely to vary with the protein and tissue type. However all invasive surgical procedures may cause local inflammation and activation of many proteolytic enzymes that are able to promote significant conformational changes including the formation of amyloid cross- $\beta$  structures. Further understanding of the principles involved in forming these aggregates that are able to contaminate surgical instruments is very important.

Studies by the researchers have demonstrated that the limited proteolysis induces the aggregation of PrP protein and the formation of Thioflavin T (ThT) positive precipitate. It was shown that the sequence of PrP between S135 and N146 is a major target for cathepsins S and L. By contrast, the proteolysis by calpain-1 produces mainly 135SAMS, a soluble fragment that does not aggregate on the same time scale as the co-precipitating mixture of 135SAMS and 144FGND. It was deduced that 144FGND is an efficient promoter of this aggregation step, apparently formed from 135SAMS. Simultaneous monitoring of the cathepsin S dependent assembly process by ThT fluorescence and of protein conformation by a newly developed method for deep UV resonance Raman (DUVRR) spectroscopy indicates that an insoluble form of proteolysed PrP loses essentially all the  $\alpha$ -helical structure (initially ~40%), and adopts a conformation with a significant proportion (~40%) of random coil, 28% of  $\beta$ -sheet and 9% of cross- $\beta$  structure.

The suspended insoluble material was also deposited onto a mica surface for AFM characterization. The AFM images show single nanoparticles with a close-to-spherical shape and large aggregates. The distribution of heights has quite sharp maximum at about 4.6 nm with periodic peaks indicating a layering assembly.

Despite a non-fibrillar morphology, the precipitated species contain a characteristic fibrillar type  $\beta$ -sheet according to a DUVRR spectral analysis. This observation may partially explain the unexpected pathological phenotype of transgenic mice expressing N-terminally deleted variants of PrP<sup>C</sup> that have a striking similarity with the fragment obtained *in vitro* by cathepsin S limited proteolysis. In addition it was found that cathepsin induced PrP aggregates were able to bind very strongly to various glass, plastic and metal surfaces and this possibly may increase the risk of the transmission of prion disease in general surgery.

### **9.1.3 Protein production and characterization.**

The group have undertaken a large study on protein production and characterization. Initially the researchers planned to look at PrP<sup>C</sup> expression and purification from *E. coli*, however, this has been extended to include eukaryotic cells (insect and mammalian cells). One of the reasons of doing this was to assess the importance and contribution of PrP glycosylation to its ability to bind metal and plastic surfaces. The researchers only used recPrP produced in their laboratory.

## **10 APPENDIX 2**

### ***Publications and conference papers by DECON Research Working Group***

#### ***CEPR, HPA***

##### ***Published papers***

Dickinson J, Murdoch H, Dennis MJ, Hall GA, Bott R, Crabb WD, Penet C, Sutton JM & Raven NDH (2009). Decontamination of prion protein (BSE301V) using a genetically engineered protease. *J Hosp Inf*, (E-pub Feb 2009).

Hesp JR, Raven NDH & Sutton JM. (2007). A role for His<sub>155</sub> in binding of human prion peptide<sub>144–167</sub> to immobilised prion protein. *Biochem Biophys Res Comm*, **362**(3): 696-699.

Murdoch H, Taylor D, Dickinson J, Walker JT, Perrett D, Raven ND & Sutton JM. (2006). Surface decontamination of surgical instruments: an ongoing dilemma. *J Hosp Inf*, **63**(4): 432-8.

Sutton JM, Dickinson J, Walker JT & Raven NDH. (2006). Introduction of methods for the decontamination of CJD-contaminated material; where to set the standard? *Clinical Infectious Disease*, **43**:757-64.

Walker JT, Dickinson J, Sutton JM, Marsh PD & Raven ND. (2008) Implications for Creutzfeldt-Jakob disease (CJD) in dentistry: a review of current knowledge. *J Dent Res*, **87**:511-9.

Walker JT, Dickinson J, Sutton JM, Raven ND & Marsh PD. (2007). Cleanability of dental instruments--implications of residual protein and risks from Creutzfeldt-Jakob disease. *British Dental Journal*, **203**:395-401.

##### ***Papers in press, under review, in preparation***

Roebuck EM, Strang R, Green I, Smith A, McLeod S & Walker J. (2008). The availability and content of dental manufacturers' decontamination information. *British Dental Journal*. (In press).

Budge C, Vassey M, Jones T, Perrett D, Nayuni N, Bennett P, Groves P, Smith A, Fulford M, Marsh PD, Raven NDH, Sutton M & Walker JTW. (2008). A quantitative assessment of residual protein levels on dental instruments reprocessed by manual, ultrasonic and automated cleaning methods. Dental instruments. *British Dental Journal*. (In preparation).

Dickinson J, Kirby E, Dennis M, Vassey JM, Smith A, Marsh PD, Walker JT, Sutton JM & Raven NDH (2008). Distribution and infectivity of prions from the mouse small intestine. *J Virol*. (In preparation).

Walker JT & Whitworth C. (2008). Cleanability of endodontic files – why they are now single-use? *Endodontic Journal*. (In preparation).

Whitworth C & Walker JT. Endodontic dentistry and vCJD. *Endodontic Journal*. (In preparation).

### **Conference posters and presentations**

Budge C, M Vassey, R Hesp, A Smith, D Perrett, J George, M Fulford, JT Walker, JM Sutton, N Raven & PD Marsh. 2007. Cleanability and removal of residual protein from dental instruments in primary care. HPA Conference, Warwick, 17-19<sup>th</sup> September 2007.

Budge CM, R Vassey, A Hesp, A Smith, D Perrett, J George, M Fulford, JT Walker, JM Sutton, N Raven & PD Marsh. 2007. Comparison of different cleaning methods used in dental surgeries. BSDR, Durham 2007.

Budge C, JT Walker, J Dickinson, AM Bennett, MJ Dennis, R Hesp, L Kirby, JM Sutton, MR Fulford, GA Hall, PD Marsh, D Perrett, AJ Smith & NDH Raven. 2006. Assessment of manual washing, ultrasonic cleaning and fully automated washer disinfectors for the removal of proteins from dental instruments. Poster. TSE Joint Funders, Warwick.

Budge *et al.*, (2006). Establishing effectiveness of cleaning methods used in dental surgeries for the removal of protein-based soils; assessment of relative performance of manual washing, ultrasonic cleaning and washer disinfectant methods. HPA Conference, Warwick.

Dickinson J, AM Bennett, C Budge, MJ Dennis, S Hanif, R Hesp, L Kirby, JT Walker, JM Wayne, JM Sutton, MR Fulford, GA Hall, PD Marsh, D Perrett, AJ Smith & NDH Raven. An evidence based study to assess the potential public health risks associated with variant Creutzfeldt-Jakob disease and dentistry. (2006). 158<sup>th</sup> meeting Society for General Microbiology (SGM), Warwick, UK 3 - 6 April 2006.

Dickinson J, A McLeod, GA Hall, MJ Dennis, H Murdoch, JT Walker, JM Sutton, NDH Raven (2005). Where do we 'set the standard' for prion decontamination? Poster presented at Neuroprion 2005, Dusseldorf, Germany.

Dickinson J, A McLeod, G Hall, M Dennis, H Murdoch, JT Walker, JM Sutton & NH Raven. (2005). Introduction of methods for the inactivation of TSE agents; a novel biological solution. USA (Abstract and Poster).

Dickinson J, Walker JT, AM Bennett, C Budge, MJ Dennis, S Hanif, R Hesp, L Kirby, JM Wayne, JM Sutton, MR Fulford, GA Hall, PD Marsh, D Perrett, AJ Smith & NDH Raven. (2005). Assessment of the transmission risks of variant Creutzfeldt-Jakob disease via dental procedures. Abstract accepted for oral presentation at HPA Conference, Warwick, UK.

Hesp JR, C Budge, JT Walker, J Dickinson, H Murdoch, JM Sutton & NDH Raven. (2006). Development and evaluation of new technologies to address the risks posed to public health by TSE diseases. CHI TSE conference, Baltimore, Washington, USA.

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## ***Igor Bronstein's Group***

### ***Published papers***

Dear DV, Young DS, Kazlauskaitė J, Meersman F, Oxley D, Webster J, Pinheiro TJ, Gill AC, Bronstein I & Lowe CR. (2007). Effects of post-translational modifications on prion protein aggregation and the propagation of scrapie-like characteristics *in vitro*. *Biochim Biophys Acta*. **1774**(7): 792-802.

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Polyakova O, Ludmila P, Dear D, Shashilov V, Bronstein IB & Lednev IK. Limited Proteolysis of Sheep Prion Protein by Cathepsin S promotes the formation of nanoparticles containing fibrillar type  $\beta$ -sheet structural motif. (*In Preparation*).

Dear DV, Young DS, Meersman F, Oxley D, Webster J, Gill AC, Bronstein I & Lowe CR. Effect of enzymatic deimination on the conformation of recombinant prion protein. (*In Preparation*).

Dear DV, Sethi R, Bronstein I & Lowe CR. Detection of recombinant prion protein binding to surgical steel - implications for contamination of surgical instruments. (*In Preparation*).

## 11 APPENDIX 3

### Profile of DH funded projects

Project Reference	Lead researcher	Establishment	Total cost	Project Title
1217034	Dr Neil Raven	HPA	£794,759	VALIDATION OF TSE DECONTAMINATION
1217035	Dr Robert Somerville	University of Edinburgh	£116,339	REMOVAL OR DESTRUCTION OF TSE AGENTS BY ADSORPTION ONTO ZEOLITE
1217041	Dr Robert Somerville	University of Edinburgh	£111,776	SURGICAL INSTRUMENT DAMAGE DURING DECONTAMINATION PROCEDURES
1217042	Dr Robert Somerville	University of Edinburgh	£1,246,780	THE RELATIVE RESISTENCE TO INACTIVATION OF DIFFERENT TSE AGENT STRAINS
1217076	Prof. John Collinge	Prion Unit	£539,689	THE DEVELOPMENT, VALIDATION, AND APPLICATION OF A MODEL TO STUDY AND IMPROVE STERILISATION OF PRION CONTAMINATED SURGICAL INSTRUMENTS
1217091	Prof. Chris Lowe	University of Cambridge	£683,077	ADSORPTION OF PRION ISOFORMS TO STAINLESS STEEL SURFACES; IMPLICATION FOR SURGICAL DECONTAMINATION PROCEDURES
1217112	Prof. David Perrett	St. Barts	£153,112	PROPOSAL TO INVESTIGATE THE EFFICIENCY OF DECONTAMINATION OF SURGICAL INSTRUMENTS AND RECOMMENDED IMPROVED PROCEDURES
1217117	Dr Igor Bronstein	IAH	£978,087	ADSORPTION OF PRION ISOFORMS TO STAINLESS STEEL SURFACES: IMPLICATION FOR SURGICAL DECONTAMINATION PROCEDURES
1217292	Prof. Chris Lowe	University of Cambridge	£198,279	ADSORPTION OF PRION ISOFORMS ON SURFACES : IMPLICATIONS FOR CONTACT LENSES AND SURGICAL EQUIPMENT
1217389	Prof. Colin Seabrook	STERIS	£746,855	PLASMA STERILISATION
0070069	Dr Robert Somerville	University of Edinburgh	£107,997	VALIDATION OF A NOVEL SURGICAL INSTRUMENT STERILISER
0070070	Dr Robert Somerville	University of Edinburgh	£278,082	NOVEL SURGICAL INSTRUMENT DECONTAMINATION PROCEDURES
0070073	Prof. Bill Keevil	University of	£505,957	DEVELOPMENT OF RAPID VISUAL

		Southampton		AND EPIMICROSCOPY TECHNIQUES
0070077	Prof. Robert Baxter	University of Edinburgh	£788,038	REMOVAL OF INFECTIVE PROTEIN RESIDUES FROM MEDICAL INSTRUMENTS
0070079	Dr Hugh Reid	Moredun Institute	£42,957	TO CREATE A VALIDATABLE PROCEDURE TO CHECK FOR THE DECONTAMINATION OF SURGICAL INSTRUMENTS
0070086	Prof. Brian Tighe	Aston University	£185,548	THE DEVELOPMENT OF A STERILE UNIVERSAL SYSTEM FOR USE WITH CONTACT DEVICES
0070088	Prof. Michael Kong	Loughborough University	£639,814	AMBIENT PRESSURE REMOVAL OF INFECTIVE PROTEIN RESIDUES FROM MEDICAL INSTRUMENTS
0070089	Prof. Eric Abel	University of Dundee	£448,577	SURGICAL INSTRUMENT COATINGS TO MINIMISE SURFACE CONTAMINATION-DIAMOND LIKE CARBON
0070090	Prof. Jim McLaughlin	University of Ulster	£138,583	PHOTOCATALYTIC COATINGS FOR DECONTAMINATION OF PRION CONTAMINATED SURGICAL DEVICES
0070099	Dr Neil Raven	HPA	£1,510,737	EXAMINATION OF THE POTENTIAL FOR TRANSMISSION OF VCJD THROUGH DENTISTRY
007/0110	Dr Mark Sutton	HPA	£383,658	EVALUATION OF OZONE STERILISATION AS METHODS FOR INACTIVATION OF TSE AGENTS

## **12 APPENDIX 4**

### ***Membership of DECON RWG 2001-2008***

1. Professor Don Jeffries (Chair)	BARTS
2. Professor Eric Abel	University of Dundee
3. Dr Alistair Aitken	University of Edinburgh
4. Dr James Barton	Herriot-Watt University
5. Dr Helen Baxter	University of Edinburgh
6. Professor Robert Baxter	University of Edinburgh
7. Dr Kostantine Borisenko	University of Dundee
8. Dr Lynne Bountiff	Food Standards Agency
9. Dr Igor Bronstein	NIMR
10. Dr Tony Byrne	University of Ulster
11. Dr Graham Cadwallader	MRC
12. Dr Gaynor Campbell	University of Edinburgh
13. Dr Kalypso Chalkidou	NICE
14. Professor John Collinge	MRC Prion Unit
15. Dr Denise Dear	University Cambridge
16. Mrs Jill Dhell	MHRA
17. Dr Joanne Dickinson	HPA
18. Ms Karen Fernie	IAH
19. Dr John Flanagan	University of Toronto
20. Dr Val Franklin	Aston University
21. Dr Howard Gee	First Water Plc.
22. Ms Joanne George	HPA
23. Mrs Louise Gibbard	Moredun Institute
24. Dr Hatem Halouani	University of Edinburgh
25. Dr Len Hazell	CSMA
26. Ms Charlotte Henley	Aston University
27. Dr Rodolphe Hervé	University of Southampton
28. Dr Richard Hesp	HPA
29. Mr Alan Hilderley	MHRA
30. Dr Jeremy Hilton	University of Ulster

31. Professor James Ironside	University of Edinburgh
32. Dr Graham Jackson	MRC Prion Unit
33. Dr Valerie Jackson	University of Ulster
34. Dr Anita Jones	University of Edinburgh
35. Professor Bill Keevil	University of Southampton
36. Mr Darryn Kerr	NHS Estates
37. Mr Tony Kinsella	CSMA
38. Dr Liz Kirby	HPA
39. Professor Michael Kong	Loughborough University
40. Dr Ian Lipscomb	University of Southampton
41. Professor Chris Lowe	University of Cambridge
42. Professor Jim McLaughlin	University of Ulster
43. Dr Edward Mackintosh	MRC Prion Unit
44. Dr Jean Manson	IAH
45. Dr Howard Mason	HSE
46. Ms Charlie Mirrieles	HPA
47. Dr Heather Murdoch	HPA
48. Professor David Perrett	BARTS
49. Dr David Perry	CSMA
50. Ms Hannah Pinchin	University of Southampton
51. Dr Frank Prior	Trust Sterile Services
52. Dr Neil Raven	HPA
53. Dr Francois Rossi	EJRC
54. Dr Fiona Russell	MRC
55. Professor Colin Seabrook	Independent Consultant
56. Dr Raj Sethi	Cambridge
57. Dr Robert Somerville	IAH
58. Dr Mark Sutton	HPA
59. Dr David Taylor	SEDECON
60. Professor Brian Tighe	Aston University
61. Dr Jimmy Walker	HPA
62. Dr Charles Weissman	MRC Prion Unit
63. Dr Gavin Whittaker	University of Edinburgh
64. Dr Qi Zhao	University of Dundee



## Department of Health Observers

Dr Iram Malik (secretariat)	DH
Dr John Stephenson	DH
Dr Elaine Gadd	DH
Mr Nigel Tomlinson	DH
Dr Peter Bennett	DH
Dr Ben Cole	DH
Dr Neil Ebenezer	DH
Dr Philippa Edwards	DH
Mr Mark Noterman	DH
Dr Emma Soto	DH

## **13 APPENDIX 5**

### ***DECON Meetings***

11 DECON RWG. Eleventh Meeting of the Research Working Group on the Decontamination of Surgical Instruments. 25 January 2005, Edinburgh.

12 DECON RWG. Twelvth Meeting of the Research Working Group on the Decontamination of Surgical Instruments. 6 July 2005, London.

13 DECON RWG. Thirteenth Meeting of the Research Working Group on the Decontamination of Surgical Instruments. 27 January 2006, Edinburgh.

14 DECON RWG. Fourteenth Meeting of the Research Working Group on the Decontamination of Surgical Instruments. 3 July 2006, London.

15 DECON RWG. Fifteenth Meeting of the Reseacrh Working Group on the Decontamination of Surgical Instruments. 16 March 2007, London.

16 DECON RWG. Sixteenth Meeting of the Research Working Group on the Decontamination of Surgical Instruments. 3 December 2007, London.

## **14 APPENDIX 6**

### ***Abbreviations***

AFM	Atomic force microscopy
AK	Adenylate kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CI	Confidence interval
CJD	Creutzfeldt-Jakob Disease
CSSD	Central Sterile Services Department
CTAB	Cetyltri-methylammonium bromide
DH	Department of Health
DLC	Diamond-like carbon
DLVO	Derjaguin, Landau, Verwey and Overbeek
ELISA	Enzyme linked immunosorbent assay
EF	Epifluorescence
EDIC	Episcopic Differential Contrast Microscopy
HPA	Health Protection Agency
HPLC	High performance liquid chromatography
IAH	Institute for Animal Health
IgG	Immunoglobulin G
i.c.	Intracerebral
i.p.	Intraperitoneal
MARS	Magnetic acoustic resonant sensor
MBH	Mouse brain homogenate
NAC	N-acetyl cysteine
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
NHS	National Health Service
OPA	o-phthaldialdehyde
PrP	Prion protein
PrP <sup>C</sup>	Cellular prion protein
PrP <sup>Sc</sup>	Disease-associated prion protein

rPrP	recombinant prion protein
SAF	Scrapie-associated fibrils
SEAC	Spongiform Encephalopathy Advisory Committee
SEM	Scanning electron microscopy
SDS	sodium <i>n</i> -dodecyl sulfate
SSD	Sterile Service Department
ToFSIMS	Time-of-flight secondary ion mass spectroscopy
TSE	Transmissible spongiform encephalopathy
XPS	X-ray photoelectron spectroscopy