Biofouling of Dental Handpieces

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Dental handpieces (HP's) are used during semi-critical and critical dental procedures that imply the HP must be sterile at the point of use. The aim of this study was to undertake a quantitative and qualitative analysis of dental HP contamination to inform the development of HP cleaning. Preliminary validation work on protein desorbtion methods and protein detection assays resulted in boiling in 1% sodium dodecylsulphate (SDS) and the o-phthaldialedhyde (OPA) assay (sensitivity 5 µg/ml) selected for further use in this study. A quantitative and qualitative analysis of HP microbial and protein contamination was then undertaken. Before decontamination, bacteria were isolated from high speed HP's (n=40) (median 200 cfu, range 0-1.9x10^4 CFU/instrument), low speed HP's(n-40) (median 400 cfu, range 0-1x10^4 CFU/instrument) and surgical HP's (n=20) (median 1x10^3, range 0-3.7x10^4 CFU/instrument). A range of oral bacteria were identified in addition to *Staphylococcus aureus* and *Propionibacterium acnes*. Protein was detected from high speed HP's (median 1.3, range 0- 210g), low speed HP's (median 15.41 µg, range 0 - 448 µg) and surgical HP's (median 350 µg, range 127.5– 1,936 µg) before decontamination. Serum albumin and salivary mucin were identified on surgical HP's before decontamination. Calcium based deposits and contaminants trapped in lubricating oil were also detected using scanning electron microscopy (SEM) and energy dispersive x-ray analysis (EDX). The efficacy of detergents and a HP cleaning solution at cleaning HP contaminants was assessed in vitro with a standard test soil and disruption of biofilms with a range of cleaning efficacies noted from each cleaning solution tested. Alkaline detergents caused a significant biomass disruption of *P. acnes* biofilms compared to ROH_2O alone. HP cleaning solution resulted in fixation of the biofilm and blood to the surface. The efficacy of novel HP cleaning machines was also assessed using a test soil based on the data generated in this study. Efficacy varied between devices tested with one demonstrating efficient protein removal in all but 1 HP location. The data presented describes a quantitative and qualitative assessment of common contaminants of HP's, mainly bacteria, salivary mucin and serum albumin. *In-vivo* biofouling levels of HP's are several fold lower than standard test soil formulations and consideration should be given to use of HP test soil based on *in-vivo* data to validate HP cleaning processes. The data generated in this thesis should aid in designing dental HP test soils and cleaning regimens.

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Acknowledgements

This piece of work is dedicated to a number of people that have enhanced my life and career.

I owe eternal gratitude to my supervisors Professor Andrew Smith and Dr Gordon Ramage for their faith in giving me this great opportunity and for their constant guideance in developing my scientific skills and completing this project. I feel I have lifelong friends as well as supervisors.

I would like to thank Alexandra Pal, Dr Kay Schloe, Charles Meadows and Gavin Knight of W & H for their support and for taking the time out to help with the project and providing me with the tools I needed. I am also greatful for the opportunity for the travel to Austria and getting to work in a completely new culture and environment. I was looked after very well and always enjoyed my visits.

I also acknowledge the contribution to my project and my life of my fellow PhD students Anto Jose, Samuel Arion Curran, Jennifer Malcolm, Sanne Dolieslager, John Paul Butcher and Leighann Sherry as well as numerous MRes students that have made the lab and the McFarlane room a fun and educational place. I couldn't have asked for a better and more supportive lab group. I never cease to be proud of us all sitting together and discussing a difficult journal club paper or all taking time out to help someone with a lab problem. The nights out weren't bad either and it could sometimes be a second to none support structure.

I would also like to thank the level 9 academic staff including Dr Shauna Culshaw, Dr Marcello Riggio, Dr David Lappin and Dr Chris Nile on level 9 who were always on hand for nights out or for scientific advice.

I would like to thank the NHS and technical staff in the department for their assistance during my project. They were sadly missed when they made the move across the road.

I am very blessed with a number of great friends of which Christopher Jenkins, Iain Dougan, Stewart McAulay, Daryl Strain, Alan Creevy, Ryan Marwaha and Andy McNicoll all deserve a mention. They have all been very understanding with the increased demands of a PhD student and I am grateful that I am always in their thoughts for any nights out, fun times and even just to meet up for a chat.

Special thanks must go to my mum Carolyn Miller my stepdad John Miller and my brother Stuart who have again been very understanding and nothing but loving and supportive to my studies throughout my life. I would also like to thank my mum for remembering who I am in those times I do manage to see her.

Equally special thanks should also go to my dad Dr Ken Smith, my stepmum Dr Gillian Lees and my step sisters Stacie and Lauren who I am fortunate enough to have not just as family members, but as scientific, legal and acountancy advisors. Their constant love and support has been a great help throughout my life and my career.

I finally must give a special mention to Miss Gi Yin Tang who has bared the brunt of my work ethic and lack of a social life. I will always be indebted to her for dragging me out for fun times even when my instinct was to have another night of writing. I think I would have gone insane without her constant love, patience and support. She is a reminder everyday of just how blessed I am in life.

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature:.....

Printed name:.....

Abbreviations

The following abbreviations are used throughout this thesis:

μg	mircrogram
μm	micrometer
μM	micromolar
ANOVA	analysis of variance
ATCC	American type culture collection
AWD	automatic washer disinfector
BA	blood agar
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CDC	centers for disease control
CDU	central decontamination unit
CFU	colony forming units
CJD	Creutzfeldt- Jakob disease
CO_2	carbon dioxide
CONS	coagulase negative *Staphylococci*
CSSD	central sterile service department
Cu	Copper
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive x-ray analysis
EFSCAN	epifluorescent scanning
ELISA	enzyme-linked immunosorbent assay
EtOH	ethylene oxide
FAA	facultative anaerobic agar
FT – IR	transform fourier infrared spectroscopy
FU	fluorescent units
g	gram
GDH	Glasgow dental hospital

h	hours
H_2O	water
HBV	hepatitis B
HIV	human immunodeficiency virus
HMDS	bis(trimethylsilyl)amine
HSA	human serum albumin
HP	handpiece
kDa	kilodaltons
LDU	local decontamination unit
M	molar
MES	2-(*N*-morpholino) ethanesulfonic acid
mg	milligram
MH	Mueller Hinton
MIC	minimum inhibitory concentration
min	minutes
ml	millilitre
mm	millimeter
mM	millimolar
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
NaOH	Sodium hydroxide
NBT	nitro blue tetrazolium chloride
NCTC	National Collection of Type Cultures
ng	nanogram
nm	nanometer
nM	nanomolar
OD	optical density
OPA	o-phthaldialedhyde
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PBST	phosphate buffered saline/tween 20
PCR	polymerase chain reaction
pg	picogram
PrP^C	host prion protein

PrP^{SC}	abnormal prion protein
PT	power tool
PVDF	polyvinylidene fluoride
RC	rich clostridium
RO	reverse osmosis
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
s	seconds
SAB	sabouraud
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SSD	sterile services department
SSS	stainless steel sections
StEM	standard error of the mean
TCA	tricholoracetic acid
ToF SIMS	time of flight secondary ion mass spectrometry
UK	United Kingdom
UV	ultraviolet
YPD	yeast peptone dextrose

1 Chapter 1: Introduction

1.1.1 *Historical Perspective*

Dentistry, the treatment of hard and soft tissues of the oral cavity, can trace its origins to the Neolithic era where evidence of flint tools used for drilling of carious teeth has been found (Bennike and Fredebo, 1985). A record of dentistry is evident throughout recorded human history, including the ancient Mayan and Egyptian civilizations. In Medieval Europe, dental barbers were the practitioners of dentistry, and not until the 18th century, with the writings of Pierre Fauchard, that dentistry became a specialised profession based on scientific principles to allow the advancement of knowledge and technique (Ring, 1985). In the modern era, dentistry is separated into eight specialities concerned with different aspects of dental surgery and practice: Periodontology, Orthodontics, Oral Surgery, Oral Pathology, Prosthodontics, Paedodontics, Dental Public Health, and Endodontics.

1.1.2 *Restorative dentistry*

Restorative dentistry is concerned with the restoration of the damaged tooth and encompasses a number of specialities. The best available methods for restorative dentistry at the turn of the century involved devitalisation of the tooth followed by the application of an ill fitting, unsightly silver amalgam or gold foil (Ring, 1985). Advancements in restoration have centred on tighter fitting implants that do not require an excess of cement to hold it in place; and the introduction of crowns and composite filling materials that provide an aesthetic, unnoticeable restoration of the tooth (Ring, 1985).

1.1.3 *Introduction and history of dental handpiece development*

An important tool that has aided the development of restorative dentistry is the handpiece (HP). The HP is a small drill that is used for the removal of enamel or carious tissue from the tooth to allow implantation. The HP provides high speed rotary power through a turbine to a metal bur for tooth drilling. Developments in the HP have allowed more intricate procedures to be performed with increased patient comfort. The history of the development of dental HP's has been reviewed extensively (Dyson and Darvell, 1993a, Dyson and Darvell, 1993b)

and the review highlights the rationale behind HP development and the many developments that have led to the development of current models. The creation of the modern HP was not a linear process and many developments occurred simultaneously such as the introduction of the fluid driven and the foot driven HP in the late 1860's (Dyson and Darvell, 1993a). The focus for further development of the HP was driven by the desire for easier control of the HP and the need for faster rotational speeds to increase patient comfort by reducing the time of operations and to take advantage of bur development (Dyson and Darvell, 1993a). These speeds could not be achieved by fluid or manually and the development of higher speed HP's capable of speeds of 10,000 revolutions per minute (RPM) occurred in 1911 (Dyson and Darvell, 1993a). The top rotational speed of HP's had increased to 100,000 RPM in 1956 and to 300,000 RPM in 1958 with the invention of the Borden Air Rotor, considered to be the first modern high speed HP (Dyson and Darvell, 1993b). The high speeds of modern HP's create a significant level of friction on the bur and the tooth surface potentially causing tooth and tissue damage. The development of cooling systems based on the spray of water onto the surface occurred in 1956 (Dyson and Darvell, 1993b). This system was inside the HP which also made control of the HP easier for the dentist. In the modern era, HP's can be subdivided by the rotary speeds of the HP and therefore the functions of the HP.

With the development of HP's, methods of sterilization have also been developed. One of the earliest reviews into dental HP sterilization was undertaken by *Appleton. J* in 1924, which described all methods that had been published and used at the time (Table 1-1). This review is of interest due to the number of methods highlighted that are suggested by authors in more recent times (Silverstone and Hill, 1999, Kolstad, 1998).

Table 1-1 Early methods of HP sterilization adapted from Appleton. J 1924.

Author and Year	Method
Witthaus (1902)	Hot air at 200°C
Anderson (1908)	Heating in mineral machine oil from 120 -150°C
Marshall (1913)	Removal of sleeve and sterilization by boiling. The rest of the instrument sterilized by placing in gasoline and storage in 95% alcohol. Alternative of dried formaldehyde vapours.
Hasseltine (1915)	Boiling water or 80°C in a water bath containing 0.25% NAOH. Excess water removed by alcohol.
Brown (1917)	Apply absolute alcohol with a cotton swab
Ash (1918)	Wiping handpiece with alcohol and sterilizing in the autoclave. Alternative of boiling in water with "a little green soap".
Schaefer (1918)	Wipe before use with a cloth wet with alcohol or a dilute phenol solution or boiled in a soap solution
Vallak (1918)	Immerses in 10% Lysol for 15 min.
Gadge (1919)	Boiled in 1% sodium bicarbonate then placed in a test tube with 95% alcohol
Appleton (1924)	Immerse completely in mineral oil at 185°C for 5 min. Wipe off oil with sterile towel.

1.1.4 *Handpiece types*

HP's can broadly be divided into high speed, low speed and surgical HP's. The high speed HP revolves at speeds of 250,000 revolutions per minute (RPM) and is used to cut hard materials such as tooth enamel, porcelain and metal fillings (Kidd et al., 2003). The development of the high speed HP with a high rotational speed was driven by the need to take advantage of developments in cutting burs and to reduce discomfort during surgery that was caused by vibrations (Cherry et al., 1974).

The modern air turbine HP can be fitted with a fibre optic light to aid visibility and a water line that allows the passage of water to cool the tooth surface and bur upon excessive heat generation (Christensen, 1999) (Figure 1-1). The high speed HP has less torque than the low speed HP; a consequence of the higher speed of rotation (Watson et al., 2000). It is for these reasons that low speed HPs are used for some procedures in dentistry. Low speed HPs can be subdivided into straight and contra angle depending on the area of access. Straight HP's are typically used for trimming temporary crowns and the contra angle HP is used for the removal of dentine or carious dentine and the drilling of pin holes (Kidd et al., 2003). Low speed HPs are capable of speeds approaching that of the high speed HP. Surgical HPs are also designed to be straight or have a contra angle for different operating areas and are less complicated to disassemble to assist in cleaning and decontamination. Surgical HPs are used to undertake a variety of procedures including wisdom tooth removal, bone modelling, apical resection and neurosurgery. The use of any HP for cutting of the tooth surface results in the generation of heat and the use of an internal spray channels allow the passage of water from the dental unit water line and onto the bur and surface to allow cooling of the surface.

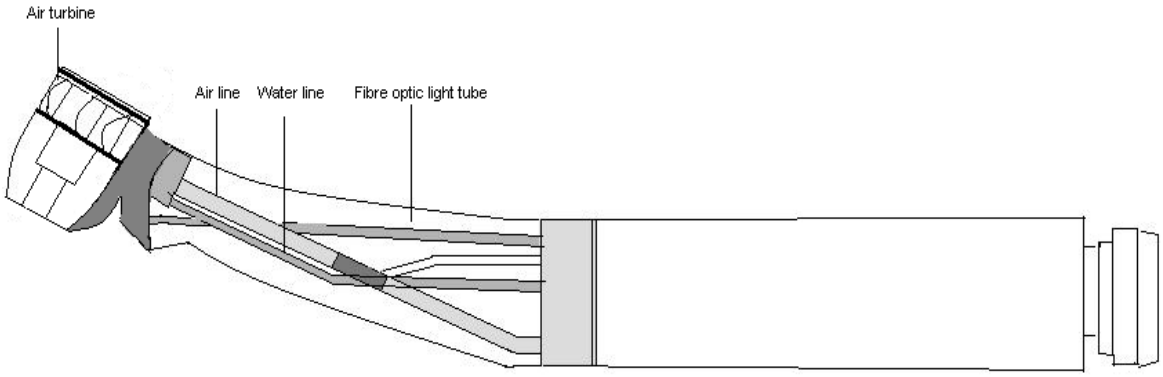


Figure 1-1 Simplified diagram of a high speed HP. The diagram displays the air line that provides rotary power to the turbine and the water line that provides water for cooling. The air drive circuit provides compressed air to drive the turbine. The shank of the bur is inserted into the turbine and revolves with it.

1.1.5 *Relationship between handpiece structure and contamination*

The workings of the HP, specifically the air derived rotary power and the spray channels for the supply of cooling water, result in contamination of the dental HP. Early studies showing internal contamination of the HP involved the visual detection of purple dye in internal sections after the operation of HP's in a purple dye solution (Checchi et al., 1998, Lewis and Boe, 1992). These studies highlighted the principal that contaminants are internalised during HP use. This dye could also be released from the HP upon further use.

1.1.6 *Internalisation of HP contaminants*

A source of contamination of any HP that utilises air is internalisation of outside contaminants. When a HP is deactivated, the deceleration of the turbine and negative pressure causes the internalisation of any external contaminant (Figure 1-2)

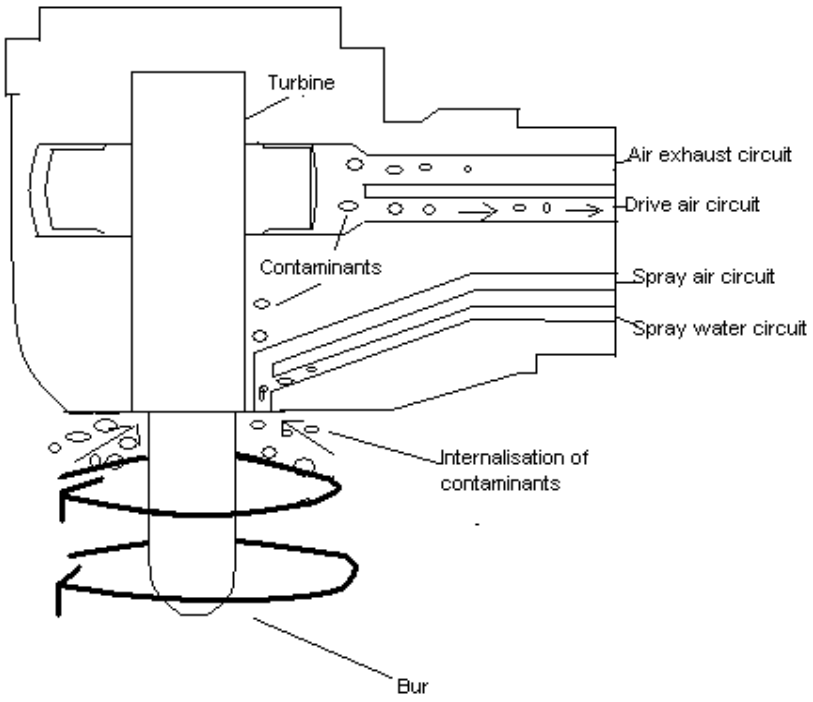


Figure 1-2 Internalisation of contaminants. Negative air pressure is created when the HP turbine is stopped which causes the internalisation of contaminants into the air and water circuits. Adapted from (Matsuyama et al., 1997)

Internalisation of contaminants results in contamination of the air line and the internal components of the HP (Lewis and Boe, 1992). The contaminants internalised are dependent on the environment and in the oral cavity may include blood, bacteria, and human tissue. Several systems and devices are common in HPs to prevent negative pressure and can result in a reduction, but not full prevention, of internalised contaminants (Hu et al., 2007b) .

1.1.7 *Dental unit waterline contamination*

The dental unit which supplies air and water to the HP has its own water supply. If this is not regularly maintained it can result in the growth of pathogenic bacteria (Montebugnoli et al., 2004). These can take the form of planktonic cells and as a biofilm on the tubing of the HP and the dental water line (Whitehouse et al., 1991). The polysaccharide matrix of the biofilm protects the bacterial cells from antibiotic agents. In a dental unit, these biofilms have been found to contain *Pseudomonas aeruginosa* and *Legionella pneumophila,* which can result in morbidity and mortality upon exposure.

The contamination of the dental unit water line and the subsequent use of water in the HP can also create an aerosol containing bacteria which may cause infection of dentists and their assistants as well as the patients (Bennett et al., 2000). Dental workers have been found to have antibodies against common contaminants of the dental unit, showing external distribution of these pathogens (Reinthaler et al., 1988).

The characterisation of contaminants of the HP, or the "biofouling", defined by the Biofouling journal as the protein, microbial and fungal contamination, has been the subject of many studies. The focus of these studies has been on the survival and transmission of bacteria and viruses.

1.1.8 *Bacterial contamination of dental handpieces*

Bacteria are detectable inside the HP structure with the contamination of air driven low speed HPs containing prophy angle attachments being studied by *Chin et al. 2006*. The aim of the study was to assess the survival and the movement of *Geobacillus stearothermophilus* between the prophy angle to the HP, and the HP to the prophy angle. The study confirmed the survival and movement of the spores from the prophylaxis angle to the HP motor on 20% of samples and from the motor to the phophylaxis angle on 40% of samples. The test spores are used for autoclave testing and do not represent a clinical contaminant or a clinical situation and for this reason the same group also assessed the presence of bacteria in low speed HP's and prophy angle attachments during HP use (Herd et al., 2007). The authors found that, the low speed HP's had a range of 0-6300 colony forming units (CFU)/ml and 75% of HP's used on 20 patients were contaminated. The swabbing method has been highlighted as inefficient by previous studies (Lipscomb et al., 2006b) and recovery rates have been found to be 19% (Angelotti et al., 1964). This may underestimate the contamination of low speed HP's in these studies.

Other studies into bacterial contamination of dental HP's was conducted by flushing decontaminated HP's and flushing HP's that had been used on patients with sterile saline through the spray channel and head of the HP (Kellett and

Holbrook, 1980). The authors isolated a range of 0-90 CFU/ml from decontaminated HP's and a range of 3.6 $x10^3$ – 29 $x10^3$ CFU/ml before decontamination. The organisms identified were similar to bacteria found in HP sprays with *Staphylococci*, including *S. aureus*, *Pseudomonas* spp and *Bacillus* spp all isolated. The flushing method employed in the study may not remove all contaminants of the HP and validation of the method would determine how much bacteria can be recovered by this method.

Bacterial contaminants of the HP can also originate from dental unit water line where it can exit the HP in the form of an aerosol spray. These aerosols are capable of spreading contaminants throughout the dental surgery. Attempts to determine the contaminants in aerosols have focused on the bacterial contaminants (Al Maghlouth et al., 2004, Rautemaa et al., 2006, Bennett et al., 2000). The bacterial content of aerosols has been identified as oral *Streptococci*, indicating contamination from the mouth, *Staphylococci* including *S. aureus* (Al Maghlouth et al., 2004), and Gram-negative *Pseudomonas* species that indicate contamination from the water line. Bennett *et al*. indicate an increased risk of respiratory problems of dental staff exposed to bacteria in the dental HP spray. Pathogenic bacteria including *Legionella* have been hypothesised to be transmitted by this route due to the association with dental treatment and the increase in *Legionella* antibodies in dental staff (Reinthaler et al., 1988).The distance of aerosol spread was assessed by *Rautemma* and colleagues by placing agar plates in different locations around dental surgeries. The study found positive cultures of bacteria in all the areas up to 2 metres away from the patient and more bacteria than is found in an empty room (Rautemaa et al., 2006).

1.1.9 *Viral contamination of dental handpieces*

The survival of pathogenic viruses in the HP has been investigated by the use of the detection of viral DNA (Hu et al., 2007b) and a bacteriophage model (Lewis et al., 1992). One study showed the contamination of high speed HPs with hepatitis B virus (HBV) (Hu et al., 2007b). The HP was operated on 40 HBV positive patients that had been split into a gingivitis group and control group. Different parts of the HP were sampled and viral contamination was determined

by the polymerase chain reaction (PCR). Viral DNA was detected in all locations sampled including the ports of the air and water line. HPs that were exposed to HBV patients with gingivitis had more isolations of viral DNA and that the use of an anti retraction valve had no effect on the presence of viral DNA. Human Immunodeficiency Virus (HIV) DNA has also been recovered from HP's after use on HIV positive patients (Lewis and Boe, 1992). The presence of viral DNA may not indicate the presence of viral particles, but indicates that HPs used on infected patients can potentially result in the contamination of the HP with viral particles. A bacteriophage model used in the Lewis and Boe 1992 study showed that whole viral particles were able to survive upon internalisation inside the HP.

1.1.10 *Contamination of surgical power tools*

The evolution of the dental HP has also resulted in the invention of other surgical power tools (PT's). Examples include surgical drills, ultrasonic dental scalers, and HP's that function using a laser. During routine use, these instruments are also contaminated with biological material (Baggish et al., 1991, Leslie et al., 2003, Sagi et al., 2002) depending on operating site. The reprocessing of surgical PT's after use is recommended by the Medical Device Directive 2002 and manufacturers of each device are required to provide validated reprocessing methods. Concerns have been raised over the effectiveness of decontamination procedures due to the complexity of some instruments and the balance struck between instrument maintenance and the elimination of contamination (Silverstone and Hill, 1999). It is important to know the location and nature of contamination in routinely used PT's before decontamination processes to understand the biological and chemical challenges to these processes. Surgical PT's can be broadly grouped into rotary, ultrasonic and laser PT's. The contaminants and the methods used to identify them may also be applied to dental HP studies.

1.1.11 *Contamination of rotary power tools*

The presence of bacteria in the exhaust air of an orthopaedic drill was highlighted by Sagi *et al*. 2002. The results showed the presence of skin

organisms such as coagulase negative staphylococci (CONS) and *S. aureus* which are associated with nosocomial infections of orthopaedic surgery (Sagi et al., 2002). Bone dust contamination during temporal bone dissection has been shown to contain neurological material which may indicate the potential for contamination with prion protein that is resistant to sterilization (Scott et al., 2001). This may occur during any neurosurgical procedure involving bone. After decontamination, studies on athroscopic shavers have detected protein and DNA contamination though this was not identified. An evaluation by Kobayashi *et al.* using energy dispersive X-ray analysis (EDX), fourier transform infrared spectroscopy and Auger microscopy revealed collagen and hydroxyl proteins after decontamination by high pressure water flow, ultrasonic cleaning, and sterilization using ethylene oxide (Kobayashi et al., 2009).

1.1.12 *Contamination of ultrasonic power tools*

Ultrasonic PT's are utilised in a variety of specialties including dentistry (Schlee et al., 2006), ophthalmology (Vargas et al., 2004), orthopaedics (Labanca et al., 2008), and neurosurgery. Applications include the removal of calculus deposits from teeth (Jotikasthira et al., 1992), the destruction of tumours the cutting of bone and soft tissue (Schlee et al., 2006) and the treatment of cataracts in the eye (Vargas et al., 2004).

The studies on the contamination of ultrasonic PTs have been concerned with contamination causing phacoemulsification endophthalmitis in patients (Leslie et al., 2003, Dinakaran and Kayarkar, 2002). This condition can lead to loss of sight of the affected eye and has been attributed to the growth of bacteria introduced through contaminated ocular solutions or PT's (Eifrig et al., 2003, Leslie et al., 2003). A study on the contamination of 32 ultrasonic PT's found bacteria, fungi, blood cells, proteinaceous material, and lens capsule fragments from previous operations in 12 of 38 PT's (Leslie et al., 2003). The contaminants were also assessed using electron microscopy. All ultrasonic PT's had been decontaminated to" hospital standards" and an automated washing system did not remove all contaminants. A further study on ophthalmic instrument contamination found debris on 3 HP's although the debris was only identified visually. A study of tip contamination with lens protein found that common lens

proteins were not detected after cleaning processes involving rinsing with "irrigating solution" and flushing with demineralised water and 70% ethanol followed by flushing with air from a high pressure pistol and sterilization by autoclaving (Nuyts et al., 1999). Protein was detected by Coomassie blue staining and Western blots for the common lens proteins αA- crystallin, vimentin, and MP26 (Nuyts et al., 1999). The lack of protein found may be due to the sensitivity of the coomassie blue technique and the specificity of Western blot for detecting specific proteins. Use of a more sensitive protein gel stain such as silver stain may result in the detection of protein. Cases of endophthalmitis have also been linked to the presence of *P. aeruginosa* that was present in the internal channels of a phacoemulsifier (Zaluski et al., 1999, Eifrig et al., 2003).

1.1.13 *Laser handpiece contamination*

Laser HP's are utilised in the field of dermatology, ophthalmology (Fine et al., 2002), and neurosurgery for the destruction of skin and tissue (Garden et al., 2002). Due to contact of the HP to the operating site during a procedure, whole cells and cellular debris has been found adhering to laser HP's after use (Wolf et al., 1991) and have been associated with the contact transmission of herpes simplex virus (Solomon et al., 2006). The formation of laser generated aerosols has been shown to transmit papillomaviruses in studies with cows (Garden et al., 2002) and would indicate the potential of transmission of human papillomaviruses. HIV DNA has been found in the aerosol but showed no infectivity when inoculated with human cells (Garden et al., 2002, Baggish et al., 1991). Viable bacteria including CONS, *Neisseria* spp and *Corynebacterium* have also been detected in aerosols in a pilot study (Capizzi et al., 1998).

1.2 Surface interaction of instrument contaminants

With knowledge of the contaminants of surgical HP's and PT's that must be removed during the decontamination process, knowledge of the interactions of these contaminants with an instrument surface may inform decontamination processes that allow removal. The adsorption of protein to surfaces is dependent on multiple factors.

1.2.1 *Protein adsorption onto surfaces*

Protein adsorption to surfaces is a problem in many areas from the dairy industry to surgical implants and medical instruments. It is possible for proteins to bind to any surface and the binding is an irreversible process (Van Tassel et al., 1998). Protein contamination of surfaces must therefore be removed through the reversal of these interactions by the cleaning process. Understanding the exact processes for protein adsorption onto surfaces can help develop the rationale for cleaning processes and develop alternative methods for the removal of protein contamination. The factors that have been determined to have an effect on protein adsorption to a solid surface are the protein structure, protein concentration, electrostatic interaction, pH of solution, hydrophobicity, and temperature of solution.

1.2.2 *The importance of protein structure*

Proteins are composed of a series of 21 amino acids linked by peptide bonds of which the arrangement is known as the primary structure (Creighton, 1993). Each amino acid can be grouped by the side chain which can be positively or negatively charged, polar neutral, or hydrophobic (Creighton, 1993). The composition of amino acids and the interactions of the side chains determine the secondary and tertiary protein structure and protein function. The secondary structure describes the number and distribution of α helices, ß sheets, and turns that are stabilised by hydrogen bonding and the tertiary structure describes the spatial relationship between all the amino acids including the formation of a hydrophobic core and the overall shape of the protein (Creighton, 1993). Proteins can be broadly placed into three categories, globular, fibrous, and membrane (Creighton, 1993).

Protein structure defines the interaction of a particular protein with other proteins, quaternary structure, and the interaction of proteins with surfaces. Protein structure can also change depending on the environment, for example an aqueous environment causes non-polar amino acid residues to reside in the interior of the protein due to the hydrophobic nature and a change in pH will cause a change in amino acid side chain charge and therefore change the

interaction of the amino acid chains (Creighton, 1993). Acidic amino residues have also been shown to have increased affinity to stainless steel and the binding of peptides can mostly be attributed to these residues (Imamura et al., 2003). Proteins can be roughly divided into “hard” and “soft” proteins depending on the internal stability of the structure which is governed by hydrophobic residues (Norde and Favier, 1995). Soft proteins are more likely to denature on a surface than hard proteins (Kubiak-Ossowska and Mulheran). Protein molecules may also undergo conformational changes that allow the most efficient flat contact binding to the surface (Kubiak-Ossowska and Mulheran).

1.2.3 *The importance of protein concentration*

An increase in protein concentration results in an increase in the amount of protein that adsorbs onto a surface (Roach et al., 2005). When the protein is present at a higher concentration, the protein adsorbed at the surface is unable to spread if another protein molecule is present in close proximity to the surface (Roach et al., 2005). At higher concentrations, more protein molecules will be present to adsorb to the surface and therefore will stop proteins spreading over a surface area (Roach et al., 2005).

1.2.4 *Effect of solution pH and temperature*

Proteins are charged molecules with the net charge being determined by the amino acid composition (Section 1.2-2). The protein net charge is also dependent on the pH of the surrounding solution with the positively charged hydrogen ions interacting with the amino acids as the pH decreases (Creighton, 1993). Altering the solution pH may reverse the interactions of the protein with the surface by causing change in both protein and surface charges. It is known that decreasing the pH below the isoelectric point of the metal oxide surface, such as that found in medical instrumentation, results in a positively charged surface which changes to neutral and finally a negative charge when the pH is above the isoelectric point (Kittaka, 1974). Therefore, an alkaline solution will, in most cases, result in a net charge of proteins and surfaces being negative which act to repel the protein from the surface (Fukuzaki et al., 1995).

Generally, an increase in temperature results in an increase in protein adsorption (Desroches et al., 2007). This effect is dependent on an increased rate of structural rearrangement of proteins. A heat denatured protein may have an increased amount of amino acid residues exposed that increase the binding to the surface (Arnebrant et al., 1986). This phenomenon is not observed for all proteins with plasma albumin showing an increase in desorption of the protein upon higher temperatures. For plasma albumin, an increase in temperature resulting in an increase in adsorption is observed when the pH is increased above the isoelectric point (Norde et al., 1986).

1.2.5 *Adsorption of prion proteins to stainless steel surfaces*

The only protein reported to cause disease is the prion protein that is the causative agent of Creutzfeldt Jacob disease (CJD) (Will et al., 1996). The prion protein is an abnormal form of the host prion protein (PrP^{C}) into the abnormal prion protein (PrP^{SC}). This causes alteration in the secondary structure including the conversion of an α helices prevalent structure to that of a ß sheet structure found in the abnormal form (Eghiaian et al., 2004). Fear of the contamination of surgical instruments by prion proteins and the subsequent transmission of CJD drives a lot of research into protein contamination of instruments (Bernoulli et al., 1977). The prion protein is also resistant to steam sterilization and the cleaning process is therefore important to remove this protein (Kast, 1976). Knowledge of the interaction of the prion protein with surfaces may inform the development of cleaning processes.

Prion protein is found to bind preferentially to nickel and molybdenum which are both prevalent in 316L medical grade stainless steel (Luhr et al., 2009). This is common with other proteins which have specific metal binding sites including human serum albumin (HSA) which has a site between two domains rich in aspartic acid and glutamic acid residues which allow for metal ion binding (Bal et al., 1998, Clarke et al., 2007). The prion protein is known to also contain metal binding sites which may allow it to behave similarly to serum albumin (Jackson et al., 2001). An increased amount of PrP^{sc} is found to adsorb to the surface with an increased drying time and an increased resistance to detergent removal (Secker et al.). This is common for other proteins as the removal of

water results in hydrophobic core amino acids moving to the outside of the protein which can increase the interaction with the surface.

Whilst the effect of prion protein structure and surface dryness is clear, there has been no research examining the effects of electrostatic interaction, protein concentration, temperature and pH on adsorption. These factors have all been shown to have an effect on protein adsorption and research into these factors may further inform the removal of prion proteins from instrument surfaces.

1.2.6 *Adsorption of bacteria to a surface*

The first event in any contamination of a biomaterial is through the adsorption of protein to the surface (Sakiyama et al., 2004). Through this, bacteria can adsorb to the proteins on the surface using specific binding factors (Tegoilia and Cooper, 2002, Piroth et al., 2008) (Figure 1-3). Bacteria have been known to initially adsorb to a surface and start producing extracellular material and form what is known as a biofilm (Costerton et al., 1978). After initial adsorption, bacteria can adsorb to the actual biofilm structure using specific receptors (Heilmann et al., 1996) A biofilm is defined as a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum, an interface or each other (Donlan and Costerton, 2002). These biofilms can form on static surfaces or in a system of flowing water such as a dental unit waterline or dental HP. Biofilms formed under a flow system are found to be more rigid and harder to remove than static biofilms which has implications for biofilm removal inside an HP spray channel which is exposed to a high flow rate (Vrouwenvelder et al., 2010).

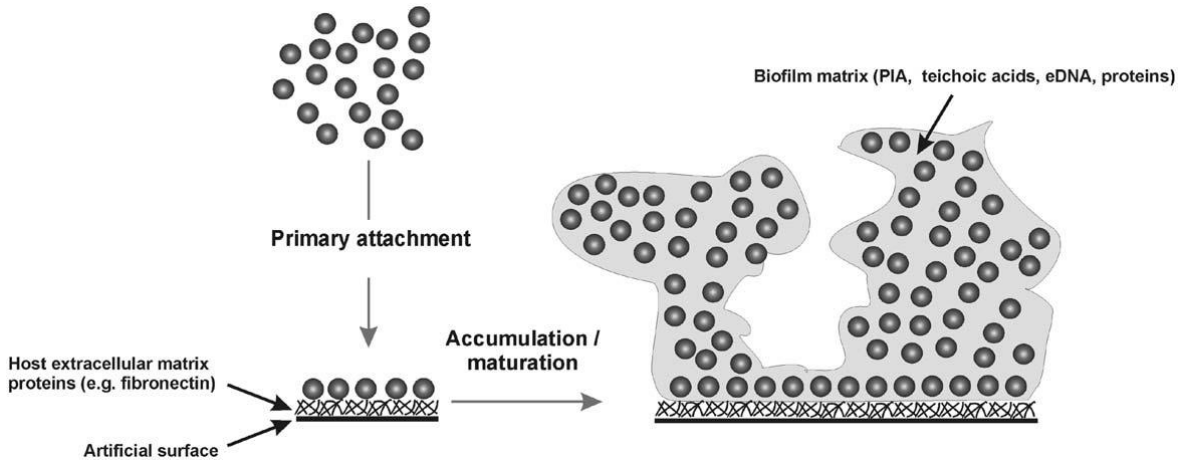


Figure 1-3 Adsorption of bacteria to a medical device. Initial binding is dependent on the presence of host proteins. Bacteria are able to bind host proteins using specific receptors and upon contact with a surface produce the biofilm ECM. Additional bacterial cells can attach to the biofilm matrix structure. Adopted from (Rohde et al., 2010).

Biofilm structure

Biofilms are composed of individual cells, extracellular matrix (ECM), and extracellular spaces (Lawrence et al., 1991). The composition and amount of exopolymeric material compared to individual cells is dependent on the species of bacteria present in the biofilm including differences with Gram negative and Gram positive bacteria (Bridier et al., Lawrence et al., 1991). Biofilms in nature tend to be multispecies depending on what bacteria are present and can adsorb to the structure (Marsh, 1994). Studies into the structure of *S. epidermidis* ECM has shown it is composed of teichoic acids, various proteins and DNA (Heilmann et al., 1996, Qin et al., 2007) whilst other bacteria such as *Escherichia coli* have been shown to produce cellulose as the main constituent (Zogaj et al., 2001) and polysaccharides are also a common constituent of ECM (Wozniak et al., 2003). All structures have a dual role of providing structural support and adsorption of bacterial cells to the matrix and the surface (Qin et al., 2007).The ECM also contains extracellular channels that allow the passage of liquid and gas all through the biofilm structure by the formation of channels (de Beer et al., 1994). This allows the transport of nutrients to cells and transport of waste products out of the biofilm allowing the survival of bacteria all through the structure (Robinson et al., 1984).

1.3 Decontamination and maintenance of dental handpieces

Invasive surgical instruments including the dental HP require decontamination after use to remove and inactivate the contaminants (Lewis et al., 1992). The decontamination of instruments requires several critical control points highlighted by the instrument decontamination circle (Figure 1-4).

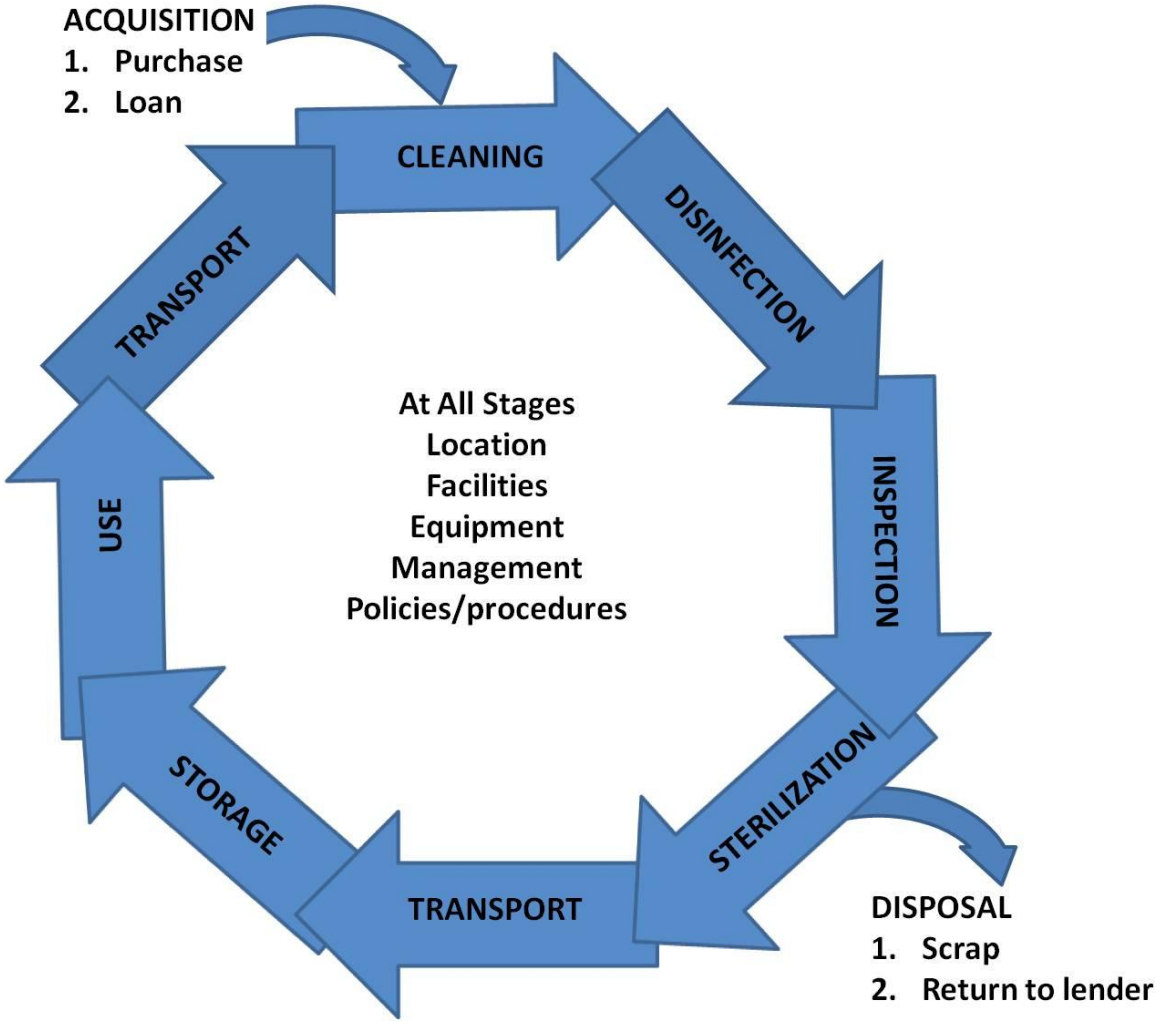


Figure 1-4 Critical control points for instrument decontamination. Dental HP's and other instruments require cleaning, disinfection, inspection and sterilization. Each critical control point is essential to the decontamination of surgical instruments. Sourced from Sterile Services Provision Review Group first report: The Glennie Framework.(NHS Scotland, 2001).

In addition, HP's require additional maintenance in the form of lubrication of the internal parts (Hegna et al., 1978). Whilst not part of any decontamination process, this ensures the continued working of the HP by reducing the friction between gear movements. Lubrication is recommended before and after decontamination by different manufacturers (Weightman and Lines, 2003) and is undertaken in the Glasgow Dental Hospital (GDH) before sterilization (Section 2.1.3). The efficacy of the cleaning and sterilization of dental HP's has been studied *in vitro* using known contaminants and *in vivo* using routine sampling.

1.3.1 *Handpiece lubricating oil*

A contaminant which is part of routine HP maintenance is the lubricant oil. The gears and the metallic parts of the HP require lubrication to prolong the life of the HP and to reduce friction during HP use. Different manufacturers recommend different lubrication procedures including before and after the sterilization process. Lubricating oil can act as a contaminant in two ways; it has been reported to protect contaminants from steam sterilization, and it can act as a contaminant itself (Lewis and Arens, 1995, Pong et al., 2005).

Lubricating oil discharge from a HP has been investigated by Pong and colleagues by visualising dyed red oil and comparing the weights of the HP before and after discharge (Pong et al., 2005). The greatest level of oil discharge was found in the first 5 minutes and a detectable amount of oil was being discharged after 40 minutes(Pong et al., 2005). The discharge of HP lubricant can have an adverse affect on dental procedures and may, in some cases, be toxic to the patient (Knight et al., 1999) . Further study of this area should focus on the discharge of HP lubricant after a typical sterilization process. A coloured dye which levels could be measured using a spectrophotometer would give quantifiable levels of oil discharge. Weight can be a variable measurement depending on the time of day that it is measured and may affect the results.

1.3.2 *Definition of terms*

The definition of each critical control point is important to determine the standards that must be attained for a product or process used in each decontamination stage. Cleaning is defined by the EN –ISO-15883-1:2006 standard as “removal of contamination from an item to the extent necessary for its further processing and its intended subsequent use”. For the validation of routine instrument cleaning, visual analysis using magnification of the instrument is employed after cleaning to ensure that no visible soil is present on the surface (Lipscomb et al., 2008). For the validation of cleaning processes, the EN–ISO-15883:2006 requires the prior soiling of instruments with various test soils and no reaction with defined protein assays to class an instrument as “clean”. The disinfection of any instrument requires a reduction, but not elimination, of microorganisms using cleaning solutions. A disinfectant solution should cause a 5 log reduction of a starting culture of 1.5 – 5x10^8 CFU/ ml *P. aeruginosa, S. aureus,* or *Enterococcus hirae* and a log reduction in viral titres of poliovirus or adenovirus upon 60 min contact time in a suspension test. Bacteria are measured using culture techniques and remaining viral titres are determined through the measurement of cell culture infectivity with remaining viral particles. Sterilization is defined by the BS- EN- ISO 11737 – 1:2006 standard as a process that results in a probability of less than 1 in a million of a single “finished” product containing a viable organism. The validation of a sterilization process relies on the inactivation of *G. stearothemophilus* spores due to the heat resistant properties of the bacterium (Ren et al.).

1.3.3 *Factors influencing cleaning efficacy and the implications of protein adsorption and biofilm formation on cleaning efficacy*

The efficacy of cleaning is determined by numerous factors detailed by the Sinners circle (Figure 1-5). These parameters include the temperature of the cleaning solution, the cleaning solution used, the amount of time that cleaning occurs for, the amount of mechanical energy that is used for the cleaning process and the water quality (Smulders et al., 2007). Altering a variable may have a detrimental effect on cleaning or may allow comparative cleaning even when other variables are changed.

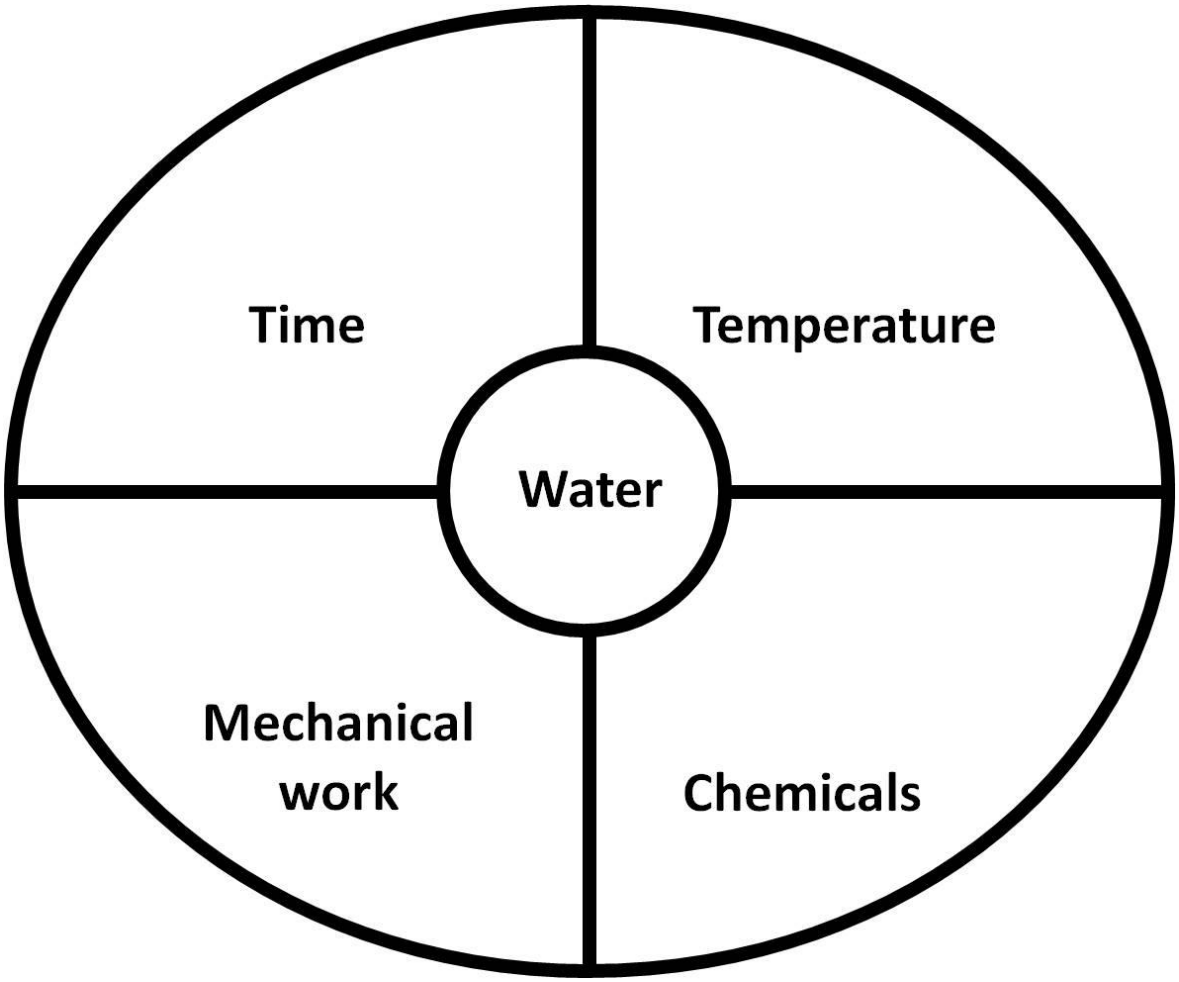


Figure 1-5 Adaptation of the Sinners circle detailing the effects of 4 variables on cleaning efficacy. Changing 1 variable can alter the need for other variables to create an efficacious cleaning process. A recent addition to the Sinners circle is the water quality. Adapted from (Smulders et al., 2007)

An increase in cleaning time and mechanical work results in a total increase in cleaning force applied to the surface. Therefore the cleaning efficacy will increase as a result. Increasing the temperature of a solution results in an increase in available energy and therefore may allow absorption energies to be overcome, however an increase in temperature also results in denaturation of proteins and may increase the rate of adsorption to the surface (Arnebrant et al., 1986). Exposing prion protein adsorbed to stainless steel wires to distilled water (H_2O) at 90°C is found to remove less prion protein than distilled H_2O at the lower temperatures of 50- 60°C (Lemmer et al., 2004). Each cleaning process must therefore involve a cleaning temperature that gives the ideal combination of increased protein removal without causing greater adsorption.

At a basic level, reversing protein interactions from a surface by cleaning occurs through dilution of the surface in a solution (Norde, 1986). If dilution with a solvent alone does not result in protein removal, the cleaning process usually incorporates various cleaning solutions or detergents of varying compositions that have different pH and recommended working temperatures (Norde, 1986). Detergents can roughly be divided into surfactants, alkaline detergents and enzymatic detergents. Alkaline detergents can both denature proteins and reverse the adsorption of proteins to surfaces by altering surface charge. Enzymatic detergents rely on various protease enzymes to breakdown the structures of contaminants. These enzymes have higher efficacy at a specific temperature and pH due to possessing specific binding sites (Lawson et al., 2007). Surfactants contain both a hydrophobic and hydrophilic parts that can denature proteins and disassociating protein aggregates. The choice of cleaning solutions is important as some have been found to fix proteinacious contamination to a surface such as glutaraldehyde and alcohol have been shown to fix proteinacious contamination such as blood to surfaces upon exposure (Prior et al., 2004, Kampf et al., 2004, Nakata et al., 2007). Whilst these cleaning solutions may be suitable for disinfecting bacteria, the solutions are not suitable for removing protein contamination.

During use, multiple proteins will adsorb to any given instrument surface each with different structures and compositions (Tsai et al., 2011). The most abundant protein will be the first to bind to the surface due to the laws of mass

transport but upon prolonged exposure these proteins may be superseded by other proteins or may have interacted with other proteins to form a complex (Desroches et al., 2007). It is therefore hard to predict the optimum cleaning process that will remove every protein and even harder to predict a reaction that would simultaneously disrupt the multiple reactions of the protein with the surface. Using a detergent may also reverse adsorption by changing the surface charges though may also denature the protein (Sakiyama et al., 2004), and in combination with a higher temperature, encourage absorption to the surface (Norde, 1986). Using an enzymatic detergent with a neutral pH may not result in protein desorption though the action of the enzyme may overcome any disadvantage associated with a neutral solution pH.

A study into the effect of different cleaning solutions has shown the effect of different chemicals and processes on the desorption of prion protein from stainless steel wires has highlighted the different modes of actions of cleaning solutions (Lemmer et al., 2004). By detecting prion protein using Western blot, it can be shown if a cleaning solution is removing the prion protein or denaturing the protein. The alkaline solutions sodium hydroxide (NaOH) and sodium hypochloride both denatured the protein and caused removal from the stainless steel surface (Lemmer et al., 2004). This effect was found to be more efficacious when at a concentration of 0.5M and temperature of 55°C is used showing the importance of combining various factors to obtain the greatest cleaning efficacy (Lemmer et al., 2004). A commercial alkaline cleaner also resulted in protein denaturation and removal (Lemmer et al., 2004). Guanidine thiocyanate, a protein denaturing agent (Suryaprakash and Prakash, 2000), was found to have no significant effect on protein denaturing or removal from the surface (Lemmer et al., 2004). A solution of 4M urea, used for protein precipitateon, also resulted in detachment but not inactivation of the protein (Lemmer et al., 2004). A sodium dodecyl sulphate (SDS) solution was found to exhibit protein denaturation and removal from the surface (Lemmer et al., 2004). A wide variety of cleaning solutions are therefore able to detach and denature prion protein and other proteins through various modes of action and the selection of detergents should be considered depending on their uses.

The formation of biofilms is associated with increased survivability of bacterial cells (Coenye et al., 2007) and decreased susceptibility to antimicrobial agents

due to the physical and chemical protection offered by the matrix (Campanac et al., 2002). The presence of biofilms on medical instrumentation presents a cross contamination issue due to the periodic shedding of bacterial cells and their retention in any complete biofilm matrix. Biofilm resistance to detergents can come from reaction of the cleaning agent with the structure or by the changes in cellular metabolism associated with biofilm growth (Campanac et al., 2002). Since proteins and biofilm can both be present on the surface, it is important to design the cleaning solution and procedure to reverse protein adsorption and disrupt biofilm structure.

1.3.4 *Current instrument cleaning methods*

Currently, the recommended cleaning processes for instrument, including dental instruments, are using a manual wash or an automated process such as a sonic bath or automatic washer disinfector (AWD). Manual cleaning has been called "the least effective method" by the *British dental journal* A12 advice sheet and Automatic cleaning processes are preferred due to their reproducibility and the ability to be validated to ensure the process is being followed consistently (Smith et al., 2009). For automated processes, the ultrasonic cleaner causes desorption of contaminants through the use of ultrasound waves passing through a liquid, which creates partial vacuum bubbles that collapse at high temperature and pressure. Since the bubbles are small in size, this only results in the removal of contamination. An ultrasonic cleaner is not suitable for dental HP's due to damage to the ball bearing gears. The AWD creates a high pressured jet of water and cleaning solution of varying temperatures and times (Table 1-2). The combination of high temperatures, high cleaning forces and the incorporation of a cleaning solution combine to clean the surface of instruments. Some AWD's also feature specialised attachments to ensure the internal cleaning of dental HP's or other instruments with narrow lumens (Walker et al., 2010).

Recent studies have shown that the AWD does not always result in the most efficacious cleaning process for all instruments when using protein contamination as a measurement (Vassey et al., 2011). The validation of cleaning processes is now considered.

Table 1-2 Details of washer disinfector cleaning stages

Manufacturer	Cleaning stages
Belimed Automated Washer Disinfector with Dr Weigert Neodisher Mediclean Fort detergent.	**Pre wash** 7 min – 25.7 °C **Wash** 15 min 51 sec – 57.7°C **1st Rinse/ 2nd Rinse** 1 min 59 sec – 57.9 °C / 2 min – 58.2 °C **Thermal Rinse** 6 min 27 sec 93.2 °C **Drying** 22 min 9 sec 90.9 °C

1.3.5 *Validation of cleaning processes*

Whilst the standard requirement of cleaning is the non detection of protein using semi quantitative protein assays, more sensitive protein detection methods have recently been described in the literature (Lipscomb et al., 2006b, Baxter et al., 2009). This has raised the issue as to how efficacious a protein assay has to be to declare that an instrument is clean (Lipscomb et al., 2006a). A total of 3 broad methods can be utilised for the inspection of instruments and the validation of cleaning, indirect, direct, and analytical methods.

1.3.6 *Indirect methods*

Indirect methods do not take a direct measurement of contamination of the surface and include gravimetric measurements, the weighing of surfaces before and after the cleaning process; ultraviolet (UV) spectroscopy that measures contaminants with an absorption spectra in the UV range and the use of an optical particle counter that can measure the size and number of particles in a solution that has been used to extract contamination from a surface. Other indirect methods involve detection of proteins by chemical reaction.

1.3.7 *Semi- quantitative protein assays*

The BS-EN-ISO-15883 standard recommends 3 semi - quantitative protein assays for the validation of reprocessing of endoscopes; the ninhydrin assay, the biuret assay, and the o – phthaldialdehyde (OPA) assay. Each of these assays reacts with a different part of the protein structure to elicit a colour change and a positive reaction with one of the assays indicates a failure in the process.

1.3.8 *Ninhydrin assay*

The ninhydrin assay involves the reaction of a ninhydrin molecule with α amino acids in the protein structure to form Ruhemmans purple (Figure 1-6) (Meyer, 1957). Ruhemanns purple can be observed visually or by measuring by spectrophotometry at an OD of 570_{nm} (Lipscomb et al., 2006b).

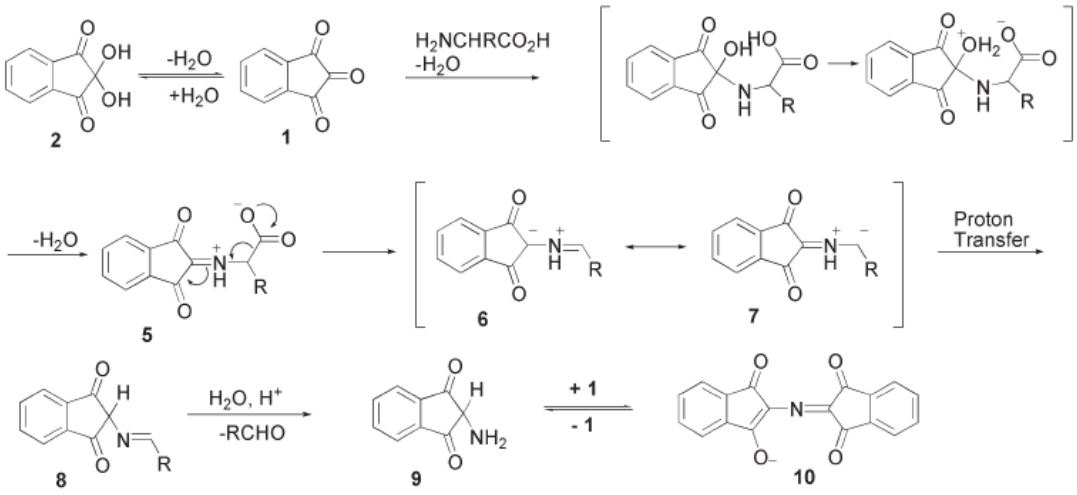


Figure 1-6 Reaction of ninhydrin molecules with amino acids forms Ruhemanns purple. Ninhydrin exists in equilibrium in a keto (1) and hydrated form (2). The Schiffs base of a reacting amine causes condensation of the central carbonyl in the keto form. The Schiffs base then forms a dipolar speies (6) and (7) by decarboxylation. The transfer of a proton then allows the formation of an intermediate aldimine (8). This aldimine is hydrolysed to an aldehyde and 2 – amino intermediate (9) which can condense with another molecule of ninhydrin to make Ruhemann's purple (10) (Friedman and Williams, 1974).

The sensitivity of ninhydrin for protein detection has shown to be 9.25 µg (Lipscomb et al., 2006a) and 5 µg when analysed in solution (Baxter et al., 2006). A ninhydin method has been utilised in a study of decontaminated instrument protein contamination which detected a range 0 – 1173 µg of protein from different instruments used for different procedures after the decontamination process (Baxter et al., 2006).

1.3.9 *Biuret and bicinchoninic acid assay*

The biuret assay involves the reduction of copper sulphate (Cu (II)) by the protein peptide backbone in an alkaline solution to Cu (I) to form a purple compound that, as with Ruhemmans purple, can be visualised or measured by spectrophotometry. Bicinchoninic acid (BCA) can be added to the reaction to chelate with Cu (I) and increase the sensitivity of the reaction of a Cu (I) – BCA violet coloured complex and an increase in sensitivity of detection (Smith et al., 1985). The biuret and the BCA reaction can be sensitive to reducing agents (Milton and Mullen, 1992), copper chelators (Walker, 1994), and hydrogen peroxide (Baker, 1991).

1.3.10 *O-phthaldialdehyde assay*

The OPA assay is based on the reaction of OPA detergent in the presence of an alkaline solution and thiol compound such as mercaptoethanol with amines of the protein or amino acid (Roth, 1971). The product formed can be detected by absorbance or fluorescence at an excitation wavelength of 338 nm and emission wavelength of 455 nm (Zhu et al., 2009). The OPA assay is not sensitive to detergents or the reducing agents and metal chelators that interfere with the BCA assay which makes it suitable for detecting protein from locations where other assays are not suitable. The OPA assay has previously been used for surgical instrument contamination studies (Smith et al., 2005).

1.3.11 *Direct methods*

The suitability of Ninhydrin and Biuret tests for the detection of contamination has come under criticism due to the lack of sensitivity (Lipscomb et al., 2006a). At the aforementioned sensitivities, neither assay would detect an infectious dose of prion protein (Lipscomb et al., 2006a). The swabbing technique has also been shown to have varying efficacies at removing contamination from a surface with as low as 19 % recovery (Angelotti et al., 1964). For these reasons, direct methods of detecting surface contamination have been proposed to increase sensitivity and to overcome the problems with sample extraction. These would allow a direct measurement of contamination on a surface rather than a small sample or extraction.

1.3.12 *Direct visualisation of contamination by behaviour of water*

The properties of water on flat surfaces can be used to detect hydrophobic films of contamination. Water will flow over flat surfaces free of these contaminating films and will gather around any hydrophobic films. This technique is not suitable for rough or ridged surfaces. The contact angle of a water droplet resting on a solid surface is influence by surface contamination. A water droplet will have a low contact angle between the droplet and surface if the surface is free of organic films, coatings and contaminants and a high contact angle of 90° when these contaminants are present (Smulders et al., 2007).

1.3.13 *Direct visual examination*

The method utilised in Sterile Service Departments (SSD's) for inspection of instrument cleanliness is a magnified visual examination of the instrument which does not detect low levels of contamination or colourless contamination (Lipscomb et al., 2006b). The use of black light will detect any contaminants on a surface that fluoresce in the presence of ultraviolet light which can detect 1 mg/cm^2 of fluorescent contaminants.

1.3.14 *SYPRO® Ruby staining and visualisation*

SYPRO® Ruby stain is composed of an organic compound bound to ruthenium as a fluorophore. The stain interacts with the amino acids lysine, arginine, and histidine (Lopez et al., 2000) which can limit binding and detection of certain proteins. The bound complex is detected using epifluorescence microscopy and has a reported sensitivity of 85 pg/mm^2 of protein (Lipscomb et al., 2006b) though this is based on operator observations. This method has been used to validate washer disinfector removal steps of prion protein (Howlin et al.) and to detect protein contamination on instruments that had been through a washer disinfector cleaning process (Lipscomb et al., 2008). The use of SYPRO® Ruby in this study highlighted the inadequacy of visual analysis at detecting low concentrations of proteinacious material.

1.3.15 *Analytical methods*

Direct and indirect methods do not identify any of the contaminants of a surface. For the identification of surface contaminants to the atomic level, a number of analytical methods are employed (Table 1-3). These methods have been used previously to study surgical instrument contamination, examining contamination in the dairy industry, and for tracing contaminants in the electronics industry. All methods described are capable of detecting atoms at varying surface depths. Examples of analytical methods are now considered.

Table 1-3 Examples of analytical methods used for cleaning validation. Taken from Handbook for Cleaning/Decontamination of Surfaces adapted from Smulders *et al.* 2007

Technique	Analysis Depth	Chemical information	Detection limit and resolution	References
X-Ray Photoelectron Spectroscopy/ Electron Spectroscopy	2-10 nm	Elemental, functional groups, oxidation state	0.1 atom % 3 μm	(Mouhyi et al., 1998)
Time of flight mass spectrometry	1-2 nm	Elemental, molecular, functional groups, isotopical	Parts per million/ parts per billion 100 nm	(Boyd et al., 2001)
Auger Electron Spectroscopy	2-10 nm	Elemental (oxidation state)	0.1% atom 10 nm	(Kobayashi et al., 2009)
Fourier transform Infrared Spectroscopy	0.5- 5 μm	Molecular, functional groups	1 % atom 0.5 μm	(Kobayashi et al., 2009)
Energy dispersive X ray analysis used with scanning electron microscopy	0.5 – 5 μm	Elemental	0.1-0.5 wt% 0.5 μm	(Baxter et al., 2006)

1.3.16 *Scanning Electron Microscopy, Energy Dispersive X- Ray Analysis and Auger Spectroscopy*

Scanning electron microscopy (SEM) produces detailed images of a surface structure and contaminants. Electrons are fired at the surface structure from a filament through a vacuum and electromagnetic lenses are used to focus the electron beam to a diameter of 100 Ä. Primary and secondary electrons scattered from a surface are collected by an electron collector and processed by a photomultiplier and videoamplifier. The current reaching the electron collector is dependent on the number of electrons that is emitted from a point in the sample. This attribute allows the generation of a light and dark image that correlates with the topography of the sample. For the identification of any contaminants viewed on a surface, SEM can be combined with EDX analysis to detect elements down to beryllium. Emitted electrons from the microscope cause ionisation of sample atoms and the ejection of an electron from the inner shell, when an outer shell electron fills the gap the excess energy is emitted as an X- ray photon and this energy can be used to characterise the atoms present. SEM and EDX analysis has been utilised in a study of decontaminated instrument contamination (Baxter et al., 2006). SEM images of reprocessed surgical instruments of varying surface complexity revealed visible contamination with EDX carbon, nitrogen, oxygen, and sulphur that indicated the presence of protein on the instruments.

A similar method for detecting surface contamination is Auger Spectroscopy. This method also relies on an electron beam ejecting electrons from the surface contaminants inner shells. An outer shell electron fills the inner shell and causes the release of an Auger electron that can be used to identify the surface contaminants. This method has been used to detect contaminants on arthroscopic shavers after decontamination (Kobayashi et al., 2009).

1.3.17 *X-ray photoelectron spectroscopy*

X-ray photoelectron spectroscopy (XPS) provides quantitative chemical information of a surface by identifying the chemical composition and the chemical states of elements. The surface is exposed to a controlled dose of X-ray energy to release photoelectrons that can be used to identify elements. If any element is bonded to another on the surface, the peak is altered to allow a more complete chemical analysis. This technique has been used to measure the cleanability of stainless steel contaminated with starch using spray cleaning and mechanical cleaning (Boyd et al., 2001).

1.3.18 *Time of flight secondary ion mass spectrometry*

Time of flight mass spectrometry (ToF – SIMS) can detect contamination to the atomic level to the order of parts per million. The ToF- SIMS technique involves the pulsing of a primary ion gallium, gold, or a bismuth ion, onto a sample surface in an ultrahigh vacuum. The collision of the primary ions with the surface results in the emission of molecules and atoms from the sample. The emitted molecules are analysed using Mass Spectrometry. ToF – SIMS analysis is best utilised with a negative control surface to put the surface analysis into context. The ToF SIMS technique has been used to examine the cleanability of stainless steel surfaces in the dairy industry (Boyd et al., 2001).

1.3.19 *Fourier transform infrared spectroscopy and Ramen spectroscopy*

Fourier transform infrared spectroscopy (FT –IR) and ramen spectroscopy identify chemical components, molecules and functional groups on a surface by measuring molecular vibrations. FT IR spectroscopy is based on the absorbance of infrared light by the sample and comparison to the absorbance profile of known molecules. Ramen spectroscopy is also based on the reaction of molecules with infrared light but involves measuring the scattering of the light by the molecule and the measurement of the scattering. FT-IR is known to be more sensitive to polar bonds and Ramen spectroscopy for nonpolar bonds.

These methods have previously been used to detect contamination of membranes in the dairy industry (Daufin et al., 1991).

1.3.20 *Implications of cleaning validation on defining cleanability*

Each cleaning validation process has differing sensitivities of protein or contamination that can be detected (Table 1-3). The European standard EN-ISO-15883 defines clean as a negative reaction with 1 of 3 protein assays (Table 1-4) implying that any protein contamination of a lower concentration is acceptable. Further work by Lipscomb et al. (2006) indicated that the minimum sensitivities of the Ninhydrin assay and the BCA assay are 9.25 µg/10mm^2 and 6.7 µg/10mm^2 respectively and protein contamination below this concentration would be acceptable. In the scientific literature, a supplement published in Zentral sterilization in 2008 for dental HP's suggests a minimum protein concentration of 200 µg/instrument which is also recommended by Murdoch et al. (2006) for surgical instruments as a level of acceptable cleanliness.To further put protein contamination into context, Lipscomb et al. (2006) state that the concentrations of 9.25 µg/ml of protein could be equivalent to 10^{14} prion infectious units and a concentration 1 µg of protein is equivalent to 10^{14} protein molecules (Baxter et al., 2006, Lipscomb et al., 2006a). Both processes require the desorption of protein from the surface before sampling, which is subject to many variables depending on the sensitivity of the extraction method, which may result in protein contamination being left on the surface (Lipscomb et al., 2006a). Due to these concerns, more sensitive methods have been developed for cleaning validation that involve direct sampling of the surface (Lipscomb et al., 2006b, Baxter et al., 2009). Both techniques can detect lower concentrations than the BS-EN-ISO -15883 standard (Table 1-4) and if these techniques were adopted as cleaning standards then an instrument protein concentration of over 85 pg/mm^2 or 0.5 µg/mm^2 would be deemed unclean and would require further reprocessing or disposal. Determining a definintion of "clean" that is achievable with current cleaning methods and be of a level to eliminate cross contamination risks is an important task for decontamination science.

Table 1-4 Cleaning validation standards described by standards and scientific literature

Study or Standard	Minimum definition of clean
BS-EN-ISO -15883 part 1	2 mg/m^2 protein detected by ninhydrin assay 30-50 µg/ml protein detected by BCA assay 0.003 µmol sensitive amino acids detected by OPA assay
Zentralsterilisation Supplement published *Oct* 2008	200 µg/dental HP
Lipscomb et al. 2006	9.25 µg /10mm^2 Ninhydrin 6.7 µg/10mm^2 Biuret test
Murdoch et al. 2006	200 µg/instrument
Baxter et al. 2009	0.5 µg/mm^2
Lipscomb et al.	85 pg/mm^2

1.3.21 *Cleaning and disinfection of dental handpieces*

A recent survey of dental HP cleaning in UK practices has found that the majority of practices wiped the external part of the HP with a cloth impregnated with disinfectant with no internal cleaning taking place (Smith et al., 2009). Internal cleaning of the HP has been validated by a study of forced air purging of the internal components of HP's which resulted in an increase in efficacy of ethylene oxide sterilization of *B. subtilis* spores and *S. mutans* bacteria (Pratt et al., 1999). No bacteria were isolated from HP's that had been purged when compared to HP's that were flushed with air and water from the dental unit alone. The study also showed that no bacteria were isolated after steam sterilization no matter what cleaning technique was employed. A herpes simplex model study was used to determine the survivability of viral particles upon disinfection (Epstein et al., 1995). The presence of viral particles was determined by examining the death of human fibroblasts upon exposure to samples from the HP. The study showed the retention of viruses in the HP after no disinfection, and external disinfection but internal disinfection of the lumens with glutaraldehyde resulted in no viral retention. Whilst glutaraldehyde is no longer appropriate for HP disinfection due to toxicity (Sagripanti and Bonifacino, 2000), this study showed the retention and expulsion of viral particles by HP's and highlighted the importance of internal disinfection in preventing cross infection. The herpes simplex model utilises an oral isolate which is an occupational dental hazard and is in the same category of germicidal resistance in the Spaulding system as HIV (Epstein et al., 1995) and is therefore not appropriate for assessing viricidal activity according to the BS-EN-ISO-14776 standard.

1.3.22 *Sterilization of dental handpieces*

Any method for sterilization of the HP must be able to be performed routinely in a dental clinic, have a short turnaround time, will not result in patient exposure to toxic chemicals during HP use, not result in damage to the HP and should deliver a sterilization efficacy detailed by BS- EN- ISO 11737 – 1:2006 in all HP locations including the internal lumens. Currently, the majority of practices in the UK utilise steam sterilization with 89% utilising a non-vacuum instrument

sterilizer and 21% wrapping the HP upon sterilization (Smith et al., 2009). Vacuum sterilizers have been recommended for HP sterilization due to the forced air removal stage removing air from the internal lumens to allow for steam penetration (Medical Devices Agency, 2006) . Vacuum sterilization also allows sterilization of wrapped instruments to ensure sterility continues until at the point of use (Weightman and Lines, 2003). Tabletop vacuum sterilizers are available and the use of wet heat ensures that the HP is not exposed to toxic chemicals. The efficacy of vacuum steam sterilizers compared to non-vacuum steam sterilizers has been shown by Andersen and coworkers (1999) by inoculating HP's with $3x10^5$ G. *stearothermophilus* and $1.4x10^6$ *Streptococcus salivarius* and sterilized HP's using 4 non vacuum and 1 vacuum sterilizer. No *G. Stearothermophilus* was detected from HP's upon sterilization using the vacuum sterilizer and growth was observed in HP's sterilized with non vacuum sterilizers (Andersen et al., 1999). *S. salivarius* was detected after sterilization using a non- vacuum sterilizer when no cleaning and lubrication had been undertaken before sterilization. Cleaning with a KaVo Rotaspray II resulted in the elimination of *S. salivarius* in 4 non vacuum sterilizers and 1 vacuum sterilizer (Andersen et al., 1999). The study highlighted the importance of cleaning before decontamination and the increased efficacy of the vacuum sterilizer though this may have been due to the wrapping of instruments for non-vacuum sterilizers which would not allow steam penetration of the instruments. The efficacy of non vacuum sterilizers on sterilizing *B. stearothermophilus* inside HP's was also assessed by *Edwardsson et al*. 1983 by inoculation of HP's in multiple locations. HP's not exposed to lubricating oil required sterilization at 134°C for 15 min to inactivate spores whilst HP's exposed to lubricating oil required sterilization times of 20 min at 134°C. Lubricating oil containing formaldehyde reduced this sterilization time to 10 min at 134°C. Steam sterilization is known to have a detrimental effect on the HP by corrosion of the instrument and alternatives to steam sterilization have been sought (Angelini, 1992, Wirthlin et al., 1981).

1.3.23 *Alternative sterilization methods for dental handpieces*

A study into sterilization of HP's was undertaken using a synthetic compressor lubricating oil heated to 150°C -160°C in a deep fat fryer (Silverstone and Hill, 1999). Oil heated to 150°C failed to sterilize *G. steraothermophilus* spores inoculated on test HP's after 75 min of heating though oil heated to 160°C resulted in sterilization of the spores after 45 min heating (Silverstone and Hill, 1999). This is significantly longer than the 3 min of sterilization currently used in steam sterilizers and will have an impact on the number of instruments available for a dental practitioner. The authors also noted the presence of oil in the HP's after "weeks" of use which may pose a health risk if oil enters the patient oral cavity. (Silverstone and Hill, 1999).

Ethylene oxide (EtOH) at room temperature has recently been suggested as an alternative sterilization method for HP's due to being less corrosive than steam sterilization due to the lower temperatures involved (Parker and Johnson, 1995, Pratt et al., 1999). EtOH was first patented as a sterilizing agent in 1937 when the antimicrobial properties of the gas were first noted (Gross and Dixon, 1937). Exposure to EtOH causes alkyation of proteins and oxidative stress to inhibit cellular metabolism and has been used to sterilize luminated devices that cannot be steam sterilized (Phillips and Kaye, 1949, Ujeyl et al., 1978). The ability of EtOH to sterilize HP's has been assessed by Parker and Johnson (1995) and Pratt et al. (1999). Parker and Johnson (1995) found that exposure to EtOH did not result in the sterilization of HP's inoculated with *Streptococcus mutans*. These HP's included unused HP's and HP's that had been in clinical practice and significantly more *S. mutans* was isolated from the HP's previously used in practice (Parker and Johnson, 1995). The authors state that the presence of protein and other contaminants may stop the penetration of gas and that it is not suitable for a sterilizing agent for HP's. *Pratt et al*. found that EtOH only sterilizes HP's if they have undergone a cleaning step with forced air purging of the internal lumens (Pratt et al., 1999). Sterilization by EtOH also requires forced air purging to ensure no gas is present in the HP and therefore requires a vacuum stage which increases the turnaround time for instruments. Due to toxicity, the EtOH device also requires trained operators and a negative pressure

room to stop gas leaks (Andersen, 1971). This may make it impractical for dental practitioners due to the expense and space issues.

The Chemiclave is a sterilization method based on formaldehyde and alcohol vapours (Kolstad, 1998). The study by *Kolstad* in 1998 showed that this method was unable to sterilize *B. stearothermophilus* in the small lumens of both low and high speed HP's and the author recommended that any dental surgery using a Chemiclave immediately change to a steam sterilizer(Kolstad, 1998)

1.4 Objectives of the study

In the light of previous knowledge of HP contamination, continuing concerns over risks of cross-contamination between patients and the emergence of variant CJD and technological improvements in understanding of medical device contamination, it is timely to re-appraise the biofouling of dental HP's after use.

The main aim of this thesis was to collaborate with a dental HP manufacturer to provide a quantitative and qualitative appraisal of HP biofouling after use in order to develop an appropriate test soil for the development of cleaning processes and prototype HP cleaning devices.

In order to achieve these aims, the work was divided into the following research questions:

1. Quantitative and qualitative analysis of microbial contamination of dental HP parts.

2. Quantitative and qualitative analysis of protein contamination of dental HP parts.

3. Propose a dental HP specific test soil

4. Undertake a detailed surface analysis of used HP parts.

5. Assessment of the efficacy of cleaning chemicals

6. Undertake assessments into the cleaning efficacy of established and prototype HP cleaning processes and devices.

2 Chapter 2: Materials and Methods

2.1 General Reagents and Equipment

2.1.1 *General reagents*

All chemicals were obtained from Sigma Aldrich (Poole, Dorset UK), and all proteomic and molecular biology reagents were obtained from Invitrogen (Paisley, Strathclyde, UK), unless otherwise stated. All blood products were acquired from E & O laboratories (Bonnybridge UK). All microbiological media was obtained from Oxoid (Hampshire UK). Reverse osmosis water (ROH_2O) was acquired from a Purelab Prima DV 34 unit (Elga water, Glasgow, Scotland). For cleaning validation studies sections made of 314l medical grade stainless steel square discs measured 10 mm by 10 mm and a thickness of 1mm were used. For epifluorescent scanning (EFSCAN) analysis, a sheet of 316l stainless steel was cut into 5mm discs and the discs were immersed in 2% (v/v) Triton X-100 detergent (Bio-Rad Hertfordshire, UK). Lubricating oil and cleaning solution used in this study was manufactured for use with the Assistina HP cleaning machine and were provided by W & H Dentalwerk.

2.1.2 *Instruments sampled in this study*

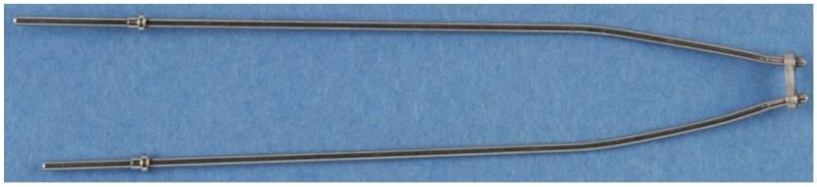
Dental and podiatry instruments sampled in this study are presented in Table 2-1, and Figure 2-1 and Figure 2-2. Dental instruments were obtained from the Glasgow Dental Hospital (GDH) central sterile service department (CSSD) and the W&H St Albans repair facility, and all podiatry instruments were obtained from podiatry clinics and the central decontamination unit at Cowlairs Glasgow. All dental HP's sampled were manufactured by W & H Dentalwerk (Burmoos, Austria) and all podiatry instruments were manufactured by Timesco Instruments (Edinburgh, UK). Upon reception to the CSSD after use, dental HP's were transported in a sterile plastic bag to a laminar flow cabinet and sampled immediately. The cap of each high speed HP was removed using a specialized tool, and the turbine (Figure 2-1) removed from the head into a sterile universal tube using a sterile pipette tip. Each low speed HP was dismantled and the spray channel (Figure 2-1) separated from the HP using a sterile pipette tip, which was then placed into a sterile 25 ml centrifuge tube. Each surgical HP was manually

dismantled and the inner gear (Figure 2-1) removed directly into a sterile 25 ml centrifuge tube. For all sampling from GDH and the repair facility, a new unused HP of each type was subjected to the decontamination process at the GDH CSSD (Table 2-3), and the parts sampled as above as negative controls. Repair facility HP's were processed on site at the facility under a bunsen burner to ensure sterility.

Table 2-1 Source and details of instruments sampled in this study.

Chapter reference	Source	Instruments sampled
3,4	GDH CSSD	TA-98 high speed HP's WA-56 Lt low speed HP's S11 surgical HP's Extraction forceps
3	St Albans repair facility	TA-98 high speed HP's TA-97 high speed HP's Topair high speed HP's WA-56 Lt low speed HP's S11 surgical HP's
4	Podiatry clinics	Pear burs Blacks files Diamond deb files

(a)



(b)

(c)

Figure 2-1 HP parts sampled in this study. The turbine from TA-98 high speed HPs (a), the spray channel from WA-56 Lt HP's (b) and gears from S11 surgical HP's (c), sampled in this study

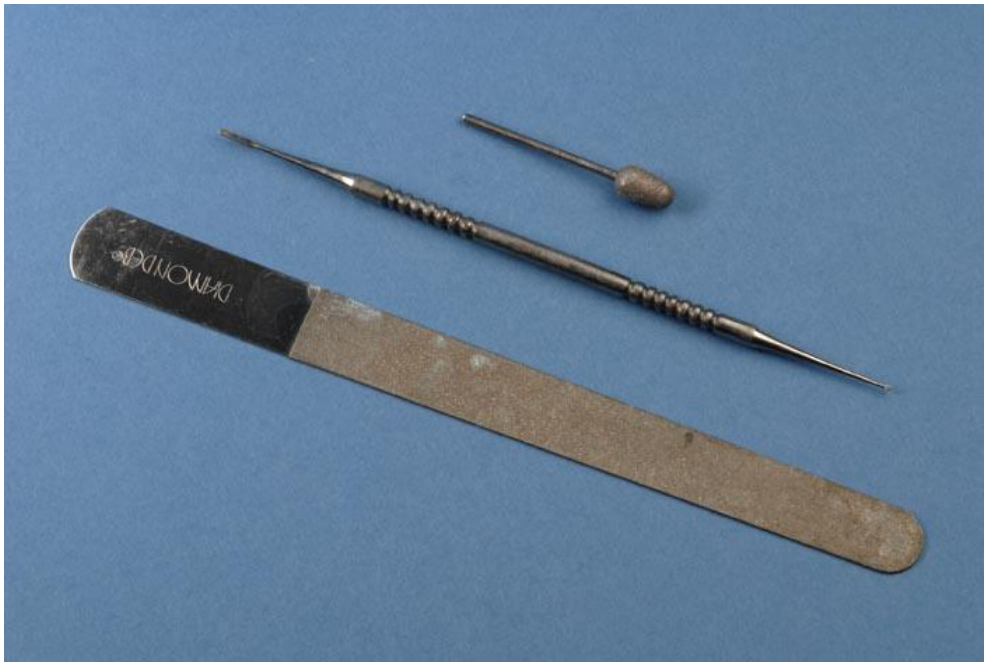


Figure 2-2 Podiatry instruments sampled in this study. Pear burs (top), Blacks' files (middle) and Diamond deb files (bottom), sampled in this study.

2.1.3 *Decontamination methods used in this study*

Instruments sampled after decontamination were subjected to a decontamination process dependent on the source of the instruments (Table 2-2, Table 2-3). For the assessment of the cleaning efficacy of novel HP cleaning machines, a total of 4 seperate processes from 2 seperate machines were sampled.

Table 2-2 Details of podiatry instrument decontamination processes

Cleaning process	Podiatry LDU	Podiatry CDU
Equipment	Hygena Ultrawave ultrasonic bath	Getinge Automated Washer Disinfector
Detergent	Sonozyme- solution changed twice daily	Dr Weigert Neodisher MediClean Forte
Cleaning time/ temperature	6 min/35˚C	Pre rinse – 4 min 38 sec/Start 31˚C End 34.9˚C Main wash - 7 min 20 sec/Start 60.5˚C, End 62.8˚C Hot water rinse – 2 min/Start 91.4˚C, End 92.6˚C Disinfection – 1 min 30 sec 37 Drying – 22 min 22 sec/Start 82.3˚C, End 87.2˚C
Validated	Tests and documentation supplied by manufacturer (Ultrawave)	Washer disinfector by trust engineer to protocols defined in SHTM2030
Sterilization Process		
Equipment	Little sister 3 Type N (Non vacuum)	Getinge Type B (Vacuum sterilizer)
Method	Steam sterilization	Steam sterilization

Table 2-3 Details of GDH CSSD decontamination process.

Process	**Equipment**	**Process**
Cleaning and Disinfection Process	**Belimed Automated Washer Disinfector with Dr Weigert Neodisher detergent.**	**Pre wash** 7 min – 25.7 °C **Wash** 15 min 51 sec – 57.7°C **1st Rinse/ 2nd Rinse** 1 min 59 sec – 57.9 °C / 2 min – 58.2 °C **Thermal Rinse (Disinfection)** 6 min 27 sec 93.2 °C **Drying** 22 min 9 sec 90.9 °C
HP maintenance	**Assistina (W & H)**	35 sec cleaning solution and lubricating oil
Sterilization	**Belimed Vacuum Sterilizer**	134 °C 3 min

2.1.4 *Cleaning solutions used in this study*

A total of 3 enzymatic, 3 alkaline detergents, an HP cleaning solution and a hand wash were used in this study (Table 2-4).

Table 2-4 Ingredients of cleaning solutions listed on MSDS sheets

Cleaning solution	Type	Ingredients
Alconox	Alkaline	Sodium Bicarbonate Sodium (C10 – C16) Alkylbenzene Sulfonate Sodium Tripolyphosphate Tetrasodium Pyrophosphate Sodium Carbonate Sodium Alcohol Sulphate
Haemosol	Alkaline	Sodium Carbonate Sodium Tripolyphosphate Urea Diethanolamine Surfactant
Rapdiex	Alkaline	Biodegradable alkyl sulphates Inorganic surface active agents Dye Alcohol
Rapizyme	Enzymatic	Surface active agents Enzymes Dyes Alcohol
Endozime	Enzymatic	2- Propanol Enzymes
Sonozyme	Enzymatic	Polyexamethylene Biguanide Hydrochloride Limonene N N- Didecyl-N-methyl-poly(oxyethyl)ammonium propionate Sodium N-lauryl B-Iminodipropionate Ethoxylated Isotridecanol
Hibiscrub	Handwash	Chlorhexidine Diacetate Hydrate
HP Cleaner	HP cleaner	N-Propanol Ethanol

2.2 Microbiological assessment

2.2.1 *Type strains used in this study*

All bacterial and fungal type strains used in this study are detailed in **Table 2-5**. These strains were used for validation and cleaning efficacy experiments.

Table 2-5 Details of bacterial and fungal strains used in this study

Bacterial or fungal strain	**Source or reference**
Staphylococcus epidermidis	National Collection of Type Cultures (NCTC) Health protection agency 11047
Escherichia coli	American Type Culture Collection (ATCC) 25922
Enterococcus faecalis	ATCC 29212
Staphylococcus aureus	ATCC 25923
Pseudomonas aeruginosa	ATCC 27853
Candida albicans	ATCC 90028
Streptococcus mutans	NCTC 10449
Streptococcus sanguinis	ATCC 10043

2.2.2 *Preparation of microbiological growth media*

All media was sterilized in a vacuum sterilizer before use and before the addition of blood products. All media was prepared according to the manufacturers instructions. For the culture of bacterial strains, blood agar (BA) was prepared by adding Columbia blood agar base and 5% (v/v) defibrinated horse blood to ROH_2O. Fastidious anaerobic agar (FAA) was prepared by adding fastidious anaerobic agar base and 5% (v/v) defibrinated horse blood to ROH_2O. Sabouraud dextrose (SAB) agar was made by adding SAB powder to ROH_2O. Mueller Hinton (MH) broth was prepared by dissolving MH powder in ROH_2O. Mannitol Salt Agar (MSA) was prepared by dissolving mannitol salt powder in ROH_2O. For the culture of fungal strains, Yeast Peptone Dextrose (YPD) broth was prepared by dissolving YPD powder in ROH_2O and Roswell Park Memorial Institute (RPMI) broth was prepared by dissolving RPMI powder in ROH_2O. Phosphate buffered saline (PBS) was made by dissolving 1 tablet (Fisher Scientific, Longborough, UK) in 100 ml of ROH_2O. To prepare agar plates, 20 ml of agar was poured into sterile Petri dishes (Sterilin, Caerphilly UK) and dried in a laminar flow cabinet for 30 min.

2.2.3 *Culture of microorganisms*

All microorganisms were maintained at -80^{o}C in Pro-tect bacterial preservers (Technical Service Consultants Limited, Heywood, UK) for the duration of the study. For the culture of bacteria, a Pro-tect bead was streaked to produce single colonies on a BA plate, or SAB agar plate for *C. albicans*, and incubated overnight at 37°C, or 30°C for *C. albicans*. For a bacterial culture, a single colony was inoculated into 10 ml of MH broth in a sterile Universal tube (Sterilin, Caerphilly UK) and incubated overnight at 37°C at 200 RPM in a KS40001 incubator (IKA™, Staufen Germany). For a *C. albicans* culture, a single colony was inoculated into 10 ml of YPD broth in a 25 ml sterile universal tube and incubated overnight at 200 RPM at 30°C in a KS40001 incubator (IKA™, Staufen Germany).

2.2.4 *Instrument sampling - standard microbial culture*

Each HP part was immersed in sterile PBS and each tube was inserted into a Fisherbrand® 11021 sonic bath (Fisher Scientific, Loughborough UK) filled with ROH_2O, and each part subjected to sonication for 5 min at 35 kHz. The instrument part was then removed and the sample retained for analysis.

2.2.5 *Identification of microorganisms*

2.2.5.1 Isolation of handpiece isolates

A 100 µl turbine eluent sample and 200 µl samples of surgical gear eluent and spray channel eluent was plated onto 2 BA plates, 2 SAB agar plates, and 2 FAA agar plates using a sterile spreader. The BA and SAB agar plates were incubated for 72 h in 5% carbon dioxide ($CO_{2)}$ in a 37°C incubator (Binder,Tuttlingen Germany). The FAA plates were incubated anaerobically in an anaerobic cabinet (Don Whitley Scientific Shipley UK) at 37°C for 7 days. Colonies isolated from BA and SAB agar plates were subcultured onto a new BA plate and SAB agar plate, respectively, to make pure cultures. Colonies isolated from FAA plates were subcultured onto a fresh BA plate and FAA plate to identify obligate anaerobes.

2.2.5.2 Gram stain of isolates

A single colony was spread on 5 µl of sterile PBS. The colony was then fixed onto a glass microscope slide and the slide was coated in 0.5% (w/v) crystal violet solution for 1 min and the slide rinsed with tap H_2O before being coated in Grams iodine for 1 min. The Grams iodine was rinsed with tap H_2O and the slide then de-stained by coating in acetone (Fisher Scientific) for 2-3 sec. The acetone was rinsed using tap H_2O and the slide coated with carbol fuschin for 1 min. The colony morphology was determined by viewing in a microscope (Olympus Essex, UK) under oil immersion at 100 x magnification.

2.2.5.3 Catalase testing of isolates

Catalase testing was performed on all Gram-positive cocci using the ID Colour Catalase test (Biomerieux Marcy l'Etoile, France) according to manufacturers' instructions. *S. aureus* was used as a positive control and *E. faecalis* was used as a negative control.

2.2.5.4 Identification of organisms by API® strip

Colonies that were identified as Gram positive cocci and catalase negative were identified using the RapidID 32 Strep API® strip (Biomerieux Marcy l'Etoile, France), according to manufacturers' instructions. Colonies identified as Gram positive bacilli were identified using API® 50C. The results of the API® strips were used to identify isolates based On the API® strip biochemical profiles.

2.2.5.5 Identification of organisms by mannitol salt agar and coagulase test

Colonies identified as Gram positive cocci and catalase positive were subcultured onto a mannitol salt agar (MSA) plates and subjected to a STAPHaurex coagulase test (Oxoid Hampshire, UK). *S. aureus* was used as a positive control and *S. epidermidis* was used as a negative control. All colonies with a positive coagulase test and a positive reaction on the MSA plates were identified as *S. aureus*. All colonies with a negative coagulase test and MSA test were identified as coagulase negative *Staphylococci* (CONS).

2.2.5.6 Identification of *P.acnes* isolates

Colonies with pyramidal morphology were identified as *P. acnes* using the Rapid ID 32A API® strip (Biomerieux Marcy l'Etoile, France). Each *P.acnes* isolate was sent for molecular typing at Queens University Belfast (McDowell et al., 2005).

2.2.6 *Instrument sampling – molecular detection (PCR)*

2.2.6.1 Processing of handpiece samples for DNA extraction

HP eluents were added into 10 ml syringes (Fisher Scientific, Loughborough, UK) and passed through a cellulose acetate filter of 0.2 µm pore size (Fisher Scientific, Loughborough, UK). The filters were then inserted into a sterile 2 ml Micro tube (Sarstedt, Leicester UK). Each filter was then immersed in 200 µl of lysis buffer (20 mM Tris-Cl, 2mM sodium EDTA, 1.2% Triton X, 20 mg/ml) and incubated at 37°C in a heat block (Grant instruments, Cambridge, UK) for 30 min. After incubation, 25 µl of proteinase K and 200 µl of Buffer AL from the DNeasy Blood and Tissue kit (Qiagen, Crawley, United Kingdom) was added to each filter and incubated at 56°C in a heat block for 30 min.

The eluent was removed and processed using the Gram positive DNA DNeasy Blood and Tissue kit extraction protocol. A total of 50 µl of AE buffer was added to the column membrane and incubated at ambient room temperature for 1h. The column was then centrifuged at 9971 *g* and a further 50 µl of AE buffer was added to the membrane. DNA concentration was then quantified using a NanoDrop (Labtech International UK, Ringmer, UK). A 1.5 µl volume of RNAse free water (Qiagen, Crawley United, Kingdom) was added to calibrate the machine followed by a 1.5 µl volume of AE buffer as a blank control. A 1.5 µl sample was then added to quantify the concentration of DNA.

2.2.6.2 Selection of primers

The 16S primers 27F (Hayashi et al., 2004) (CAGGCCTAACACATGCAAGTC) and 1387R (Marchesi et al., 1998) (GGGCGGWGTGTACAAGGC) with annealing temperatures of 60°C were utilised. A basic local alignment search tool (BLAST) search of the sequences was performed on the NCBI website to ensure sequence homology with the 16S ribosome.

2.2.6.3 16S Polymerase Chain Reaction

Each DNA sample was diluted to 1 ng/ml in DNAse free H_2O (Sigma, Dorset UK). A positive control was also diluted and a negative control of DNAse free H_2O was also processed. A 1 µl sample of DNA was added to 22.8 µl of ReddyMix™ and 0.1 µl of each primer 27F and 1387R both at concentrations of 50 µM/µl. The reaction tubes were inserted into a PCR MyCycler™ (Bio-Rad Hertfordshire, UK) and operated for one cycle of 95°C for 2 min followed by 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min 30 sec and an extension cycle at 72°C for 10 min.

2.2.6.4 Agarose gel electrophoresis

A 5 µl solution of 10 mg/ml of ethidium bromide was added to a 100 ml 2 % (w/v) agarose gel. Each sample was added to a well of the gel and 2 µl of 100bp ladder (New England Biolabs) was added for size comparison. The gel tank was plugged into a BioRad power pack and operated at 100v for 60 min. The gel was photographed in a BioRad XR+ Gel doc using ultraviolet light.

2.3 Protein quantification and analysis

2.3.1 *Quantitative protein sampling*

2.3.1.1 Bicinchoninic acid assay

The bicinchoninic acid (BCA) reagent was prepared by adding 1 part of the BCA solution B (Pierce Biotechnology, Rockford USA) to 50 parts of BCA solution A (Pierce Biotechnology, Rockford USA). For determination of protein concentration, 100 µl of protein sample was added to 1 ml of the BCA reagent. Samples were incubated for 1h at 60°C in a water bath (Grant Instruments, Cambridgeshire, UK) and were then cooled to room temperature. A 300 µl sample was added to a Costar™ clear, flat bottomed 96 well plate and all samples were analysed using a Sunrise™ plate reader at OD 570.

2.3.1.2 Ninhydrin assay

For the ninhydrin assay, a 500 µl sample of 2% (v/v) ninhydrin solution was added to 500 µl of protein sample. Samples were incubated for 10 min at 60^{o}C, and were cooled to room temperature. A 300 µl sample was added to a Costar™ clear, flat bottomed 96 well plate and all samples were analysed using a Sunrise™ plate reader at OD 570 (Starcher, 2001).

2.3.1.3 Ophthaldialdehyde assay

The OPA reagent was prepared by adding 40 mg of phthaldialdehyde dissolved in 1 ml of methanol (BDH Laboratory Supplies, Leicester, UK) to a solution of 100 mg of mercaptoethanesulfonate dissolved in 50 ml of 0.1M pH 9.2 sodium tetraborate. A 20µl protein sample was added to a Costar™ dark flat bottomed 96 well plate (Sigma, Dorset, UK) and 300 µl of OPA reagent was added to each sample. The samples were incubated for 3 min at ambient room temperature before being sampled using an Omega Fluostar plate reader (BMG Labtech, Aylesbury UK) at excitation wavelength 355 nm and emission wavelength 460 nm(Zhu et al., 2009).

2.3.2 *Protein sample precipitation*

2.3.2.1 Precipitation of protein using acetone

Four volumes of -20°C acetone was added to 1 volume of protein solution and incubated for 1 hour at -20°C. The samples were then centrifuged at 2215 *g* in a MSE Centaur 1 centrifuge (Sanyo, Loughborough, UK) for 35 min and the protein pellet re-suspended in 1 ml of 1% (v/v) SDS (Jiang et al., 2004).

2.3.2.2 Precipitation of protein using Trichloroacetic acid

Trichloroacetic acid (TCA) solution was made by adding 500 grams of TCA powder to 350 ml of ROH_2O. For precipitation, 1 volume of TCA solution was added to 4 volumes of protein sample and incubated for 10 min at 4°C. The samples were centrifuged at 9971 *g* for 5 min and the supernatant removed. The protein pellet was then washed in 200 µl of ice cold acetone and centrifuged at

9971 *g* for 5 min. The pellet was washed a further two times in acetone and the samples were added to a heat block (Grant, Cambridge UK) at 95°C to drive off the remaining acetone (Jiang et al., 2004). The pellet was then resuspended in 1 ml of 1% (v/v) SDS.

2.3.2.3 Precipitation of protein using StrataClean™ Resin

A 10 µl aliquot of StrataClean™ resin (Agilent Technologies, Texas USA) was added to 1 ml of protein solution and centrifuged at 614 *g* for 1 min and the supernatant removed (Koch-Nolte et al., 1996). The resin pellet was resuspended in 1 ml of 1% SDS (v/v) or NuPAGE™ sample buffer.

2.3.2.4 Precipitation of protein using an Amicon® filter

Amicon® 15 K filter units were acquired from Fisher Scientific Longborough UK. Protein samples were loaded into the Amicon® filter unit and centrifuged at 2215 *g* for 45 min according to manufacturers instructions. The retentate and the filtrate were both removed for analysis.

2.3.3 *Sodium dodecyl sulphate polyacrylamide gel electrophoresis*

Samples were mixed with an equal volume of NuPAGE™ sample buffer and heated for 10 min at 70°C. Each sample was then centrifuged in a MSE Microcentaur (Sanyo Loughborough, UK) at 9971 g for 2 min. Running buffer was made by diluting 20 x NuPAGE® 2-(*N*-morpholino) ethanesulfonic acid (MES) running buffer to make a 1 x solution. For the sampling of salivary mucin, running buffer was made by diluting 20 x NuPAGE tris-acetate buffer to make a 1x solution. An XCell SureLock Mini-Cell™ gel case was filled with 200 ml of running buffer in the inner chamber and 600 ml of running buffer in the outer chamber. For the detection of salivary mucin, a pre-cast 3-8% NuPAGE® Novex® Tris Acetate gel and for all other sampling a pre-cast 4-12% NuPAGE® Novex® Bis Tris gel was used. Each gel was inserted into the tank and the wells washed 3 times with running buffer. A maximum of 20 µl samples were loaded into each

well and 0.5 µl of BenchMark™ protein ladder was loaded for size comparison. The gel tank was connected to a Powerpac 300 power pack (Bio-Rad, Hertfordshire, UK) and operated at 200V for 35 min for Bis Tris gels and 150v for 1 h for Tris -Acetate gels.

2.3.4 *Gel staining*

2.3.4.1 Staining of gel by Coomassie Blue

Coomassie blue solution was made by dissolving 0.25 g of coomassie brilliant blue G- 250 powder per 100 ml of methanol: acetic acid solution comprising 500 ml of methanol, 400 ml of ROH_2O and 100 ml of glacial acetic acid (BDH Laboratory Supplies, Leicester UK) (Sambrook. J and Russel. W.D, 2006). For staining, the gel was immersed in 100 ml of coomassie brilliant blue solution on a rocking platform for 4 h at room temperature. The gel was then destained in a methanol: acetic acid solution until bands were visible (Sambrook. J and Russel. W.D, 2006). Gels were photographed in a XR+ Gel doc (Bio- Rad, Hertfordshire UK).

2.3.4.2 Staining of gel by SYPRO® Ruby

The gel was removed from the outer casing and immersed in 100 ml of SYPRO® ruby stain for 3 h before being destained in 100 ml of a solution of 10% (v/v) methanol and 7% (v/v) acetic acid in ROH_2O for 1 h. The gel was viewed using an XR+ Gel doc using ultraviolet light (Bio- Rad, Hertfordshire, UK).

2.3.4.3 Staining of gel by Silver Staining

The gel was removed from the outer casing and stained using the Silverquest™ silver stain kit according to the manufacturer instructions. Gels were imaged in a XR+ Geldoc (Bio-Rad, Hertfordshire, UK).

2.3.4.4 Staining of gel by periodic acid Schiff reagent

For periodic acid Schiff (PAS) staining, the gel was removed from the outer casing and immersed in 100 ml of fixative solution comprising a 40% (v/v) ethanol and 7% (v/v) acetic acid in ROH_2O for 30 min. The solution was then replaced and the gel fixed overnight. The gel was then immersed in fresh fixative solution 4x for 30 min with fresh fixative solution each time. The gel was then immersed in a solution of 1% (w/v) periodic acid and 3% (v/v) acetic acid for 60 min before being washed 10x for 10 min in ROH_2O. The gel was then stained with Schiff's Reagent for 60 min in the dark. Gels were photographed in a XR+ Gel doc (Bio-Rad, Hertfordshire, UK).

2.3.5 *Mass spectrometry data analysis*

Each protein band was sent for mass spectrometry (MS) analysis at the Glasgow University Proteomics facility. The MS/MS spectra were used for interrogation of the human and bacterial proteome database using Mascot software (http://www.matrixscience.com). A protein was considered a good identification if 2 or more peptides had an individual ion score over 58 which indicates significant homology ($p<0.05$) to a protein listed in the database (Romero et al., 2010).

2.3.6 *Western blot*

Transfer buffer was prepared by dissolving 10 mM Tris base, 100 mM glycine, and 25 ml methanol in 975 ml of ultrapure ROH_2O. Blocking solution was made by dissolving 10% non-fat milk powder (Malvern St Albans UK) in Tris buffered saline (TBS) buffer (50 mM Tris- HCl, 150 mM NaCl). Wash buffer was made by dissolving 0.1% Tween® 20 in Tris- ethylenediaminetetraacetic acid (EDTA) -NaCl buffer (25 mM Tris base, 1 mM EDTA, 150 mM NaCl). A solution of 5-Bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium (BCIP NBT) was made by dissolving a SIGMAFAST™ BCIP NBT tablet in 10 ml in ultrapure H_2O.

Each sample was sampled by SDS PAGE (Section 2.3.3) and all gels included a 10µl sample of MagicMark™ XP pre-stained protein ladder for size comparison. The gel case was opened and a piece of filter paper pre- soaked in transfer

buffer was then placed on top of the gel. The PVDF membrane was soaked in 100% methanol for 30 sec and then immersed in transfer buffer for 3 min. The membrane was then placed on top of the gel and air bubbles removed. A piece of pre soaked filter paper was then placed on top of the membrane and 2 blotting pads, soaked in transfer buffer, were placed on both sides of the membrane sandwich and the assembly was added to the X- cell II™ Blot Module. The blot module was inserted into an Xcell SureLock Mini-Cell™ and the membrane assembly was immersed in 200 ml of transfer buffer and the outer core was filled with 600 ml of ROH2O. The gel tank was connected to a power pack (Bio-Rad, Hertfordshire, UK) and operated at 30V for 1 h. The membrane was immersed in 100 ml blocking solution and incubated for 1 h on a tilting platform. The membrane was then inserted into a 50 ml Corning centrifuge tube and immersed in a 10ml solution of primary antibody diluted in wash buffer and 1% (v/v) blocking solution. Each membrane was incubated for 1 h at 37°C in a hybridisation oven (Hybaid Thermo Scientific, Leicestershire, UK and washed or 4x 5 min in wash buffer. The membranes were then inoculated with 10 ml of secondary antibody for 1 h in the hybridisation oven at 37°C. The membranes were then washed a further 4 x for 5 min in wash buffer. Each membrane was then immersed in 10 ml of BCIP NBT solution and incubated until the alkaline phosphatase representing bound antibody was visible. The membranes were imaged using an XR+ Gel doc (Bio- Rad, Hertfordshire UK) and the presence of protein bands in each lane was determined using Quantity one® gel interpretation software (Bio Rad version 4.6.7).

2.3.7 *Indirect enzyme linked immunosorbent assay*

Samples and standards for the enzyme linked immunosorbent assay (ELISA) were diluted in PBST. For the ELISA, a 100 µl sample of 1:1000 dilution of capture antibody (Pierce biotechnology Rockford USA) was added to each well of a Immuno 96 Microwell™ clearplates (Fisher scientific Longborough, UK) and incubated at 4°C overnight. The plate was then given 5x washes in PBS and 10% tween 20 (PBST) and incubated in blocking solution at room temperature (5% milk powder in PBST) for 1h. The plate was then given 5x washes in PBST and the samples were then added to the plates and incubated for 90 min at room temperature. The plate was then given 5x washes in PBST and a 100 µl sample of

1:1000 dilution of secondary antibody was added to the plate and incubated at room temperature for 90 min. The plate was then given 5x washes in PBST and a 100 µl sample of a 1:100000 dilution of the detection antibody was added to each well and incubated for 90 min at room temperature. The plate was then given 5x washes in PBST and a 100 µl sample of tetramethylbenzidine was added to each well. The plate was incubated in the dark for 30 min and when a blue colour had developed, the reaction was stopped by 100 µl of 0.12M of HCl. The plate was then read in an Omega Fluostar plate reader (BMG Labtech, Aylesbury UK) at an absorption wavelength of 450 nm and a reference wavelength of 630 nm.

2.4 Surface analysis

2.4.1 *Surface analysis techniques*

All 316l stainless steel discs sampled were cleaned by sonication at 35kHz for 15 min in 1% Triton X and then subjected to 15 min sonication in HPLC grade RO H_2O (Fisher Scientific Longborough, UK) to ensure no residual contaminants were present. The discs were then dried in a drying cabinet at 37°C.

2.4.1.1 Scanning electron microscopy

For fixing, the sample was immersed in fixative, comprised of 2 % paraformaldehyde, 2 % gluteraldehyde, 0.15M sodium cacodylate, and 0.15 % alcian blue, for 22h. The fixative was then removed and replaced with 0.15M sodium cacodylate. The samples were then given 3 further washes in 0.15M sodium cacodylate buffer for 5 min per wash before immersion in a 1:1 solution of 1% osmium tetroxide: 0.15M sodium cacodylate for 60 min. The samples were then rinsed 3 times for 5 min in distilled water and immersed in 0.5 % uranyl acetate for 60 min in the dark. The samples were then rinsed in distilled water and then dehydrated by immersion 2 times for 5 min in 30% alcohol, 2 times for 5 min in 50% alcohol, 2 times for 5 min in 70% alcohol, 2 times for 5 min in 90% alcohol, 4 times for 5 min in absolute alcohol and 2 times for 5 min in dried absolute alcohol. The samples were then immersed in hexamethyldisilazane (HMDS) 2x for 5 min. The samples were moved to a new 24 well plate and placed

in a dessicator overnight to allow the sample to dry. Each sampled to be viewed was sputter- coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope at 12 x and 100 x magnification.

2.4.1.2 Energy dispersive X-ray analysis

For EDX analysis, images of parts were taken using a Philips XL30CP instrument operating at 20 kV. For any surface contaminants, EDX analysis was performed using an integrated Isis 300 X-ray analyser (Oxford instruments, Oxford, UK). Each contaminant was subjected to elemental analysis to a depth of 3 µm using an X- ray fluorimeter capable of detecting elements of atomic number greater than 6.

2.4.1.3 Epifluorescent analysis

EFSCAN analysis was undertaken at the University of Edinburgh chemistry department in collaboration with Dr Helen Baxter and Professor Robert Baxter. Each part was mounted on a computer controlled motorized x-y translation stage (Ocean optics, Ostfildern Germany) and each part excited at 468nm fibre optic diode (Richardson et al., 2004). The fluorescent signal was measured using a photon counting fluorescence spectrometer (Jobin-Yvon-Horiba Fluoromax-P) and the data analysed using the LabView program (National Instruments, Austin TX, USA) (Baxter et al., 2009, Richardson et al., 2004)

2.5 Surface cleaning studies

2.5.1 *Optimising cleaning parameters*

2.5.1.1 Preparation of stainless steel sections

Each stainless steel section (SSS) was immersed in 0.1M sodium hydroxide (NaOH) pH 9.2 and boiled at for 10 min at 100°C in a water bath (Grant instruments) to remove residual contaminants from the discs. The discs were

then rinsed with methanol (BDH laboratories, Leicester UK) and dried in a laminar flow cabinet for 1 h (Imamura et al., 2003).

2.5.1.2 Test Soil

The test soil used was the Swedish test soil detailed by the BS EN ISO–15883-5: 2006. The soil was made by adding 1 ml of 0.1M calcium chloride ($CaCl_{2)}$ (Difco Oxford UK) to 9 ml of citrated horse blood (Ransjo et al., 2001).

2.5.1.3 Inoculation of stainless steel sections

A total of 3 SSS's were inoculated with 30 µl of solution of recalcified horse blood. SSS's were inoculated with 30 µl of 0.1M $CaCl_2$ for negative controls. Each inoculated SSS was dried for 16 h at ambient room temperature representing the time it can take for instruments to be reprocessed in a sterilization department (Plinston et al., 2007). Each disc was inserted into a separate well on a clear 24 well plate. For each experimental run, the control comprised a protein assay for 30 µl of citrated blood diluted in 1ml of RO H_2O. The percentage removal was expressed as the amount recovered from the experiment wash compared to the protein detected in the control.

2.5.2 *Cleaning system*

The 24 well plate containing inoculated stainless steel discs was placed on the measured centre of a Grant PMR – 30 rocking platform. Each well containing a disc was filled with 1 ml of the appropriate cleaning solution. The experiment consisted of 3 discs and the experiment repeated 3 times.

2.5.3 *Biofilm cleaning model*

2.5.3.1 Culture of microorganisms

A total of 20 *P. acnes* strains isolated from used, unprocessed s11 W &H surgical HPs from the Glasgow dental hospital (GDH) and 19 *P. acnes* isolates isolated from decontaminated s11 surgical HPs from the St Albans repair facility were revived from Protect beads (Biomerieux Marcy l'Etoile, France). A single bead

was plated onto FAA and incubated anaerobically for 24 h at 37°C. *S. epidermidis* strain (RP62A) was cultured onto BA and incubated overnight at 37°C under 5 % CO_2. A single colony of each *P. acnes* strain and the *S. epidermidis* was added to 50 ml of reinforced clostridial (RC) broth that had been pre-reduced by boiling for 30 min. Of the GDH isolates, a total of 13 cultures were incubated overnight at 37°C anaerobically and 7 cultures were incubated overnight aerobically at 37°C. Of the repair facility isolates a total of 11 cultures were incubated overnight at 37°C anaerobically and 8 cultures were incubated overnight at 37°C aerobically.

2.5.3.2 Preparation of biofilms

The OD of each culture was taken using a spectrophotometer (Fisherbrand Longborough, UK). Each culture was diluted in RC broth to an O.D of 0.2 representing $1x10^8$ CFU of bacteria. A total of 6, 300 µl samples of each isolate were added to separate wells of a clear Costar 96 well flat=bottomed plate (Sigma Aldrich, Dorset UK). Separate plates were used for each cleaning solution. Each plate was placed on a PMR tilting platform (Grant Instruments Cambridge, UK) at moderate speed (18 rpm) and incubated for 72 h anaerobically at 37°C or for 16 h aerobically at 37°C. RC media was added to each plate as a negative control. RC broth was changed every 24 h in each well for the 72h biofilms.

2.5.3.3 Detergent treatment of biofilms

For 72 h biofilms, a 1% (v/v) solution of Haemo-sol® detergent and undiluted W & H cleaning solution were sampled. For 16 h biofilms, a 1% (v/v) solution of Alconox®, Haemo-sol® and Rapizyme® and an undiluted W & H cleaning solution were sampled. The media was removed from each well and the plate air dried for 20 min. Each well was washed three times in sterile phosphate buffered saline (PBS) and a 300 µl sample of the appropriate cleaning solution was added to each well and incubated at room temperature for 16 min, which is the length of time an instrument is exposed to detergent during some automated washing process. After exposure, the supernatant was removed and the wells rinsed a further 3 times in sterile PBS. Samples were compared to a positive, untreated

control and a positive control treated with PBS in place of a cleaning solution. The media was removed each day and cultured on FAA plates as controls.

2.5.3.4 Biofilm biomass sampling

A total of 300 µl of 1% (w/v) crystal violet solution was added to each well and incubated at room temperature for 10 min. The wells were rinsed 3 times by tap water and 300 µl of a 70 % ethanol/5 % acetone solution was added to each well. The plate was incubated for 15 min and the solution transferred to a fresh plate before being read in a plate reader (Sunrise™ Tecan) at OD_{570}.

2.5.3.5 Biofilm bacterial cell viability

The bacterial cell viability of 16 h biofilms was measured using the alamarBlue® assay (Invitrogen Strathclyde, UK) according to manufacturer's instructions. Each plate was incubated for 4 hours aerobically at 37°C and the plate measured in an Omega Flurostar plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

2.6 Data analysis

Data was entered into Microsoft excel and all calculations were carried out using the excel program. All data to be converted to graphs was entered into GraphPad Prism 4. Statistical analysis was carried out using GraphPad Prism 4 unless otherwise stated.

3 Chapter 3: Assessment of Assays for Detection of Protein Contamination

3.1 Introduction

Invasive surgical instruments are contaminated by protein during routine use that may inhibit instrument sterilization (Nakata et al., 2007). Residual protein must therefore be removed by cleaning, the critical control stage of the decontamination process before sterilization can take place (Alfa et al., 2006, Weightman and Lines, 2003). It is for this reason that protein contamination is used as a measurement for the efficacy of cleaning processes, and the European standard ISO–EN-15883:2006 part 1 provides details on the construction of test devices, test soils and protein detection methods to assess the efficacy of washer disinfectors. The protein detection methods named in the standard are the ninhydrin assay, the BCA assay, and the OPA assay. Each assay binds to a different part of the protein structure to create different measurable products. The ninhydrin assay is based on the binding of a ninhydrin molecule to an α amino acid in a protein to produce Ruhemman's purple which can be detected using spectrophotometry (Meyer, 1957). The BCA assay is based upon the reaction of a protein peptide backbone with copper sulphate in an alkaline solution (Smith et al., 1985). This process is more sensitive when bicinchoninic acid is added to chelate with cupric sulphate reduced by the peptide backbone (Smith et al., 1985). The third recommended method is the reaction of OPA detergent, in the presence of a fluorescent reagent such as mercaptoethanosulphanate, with amines in the protein known as the OPA assay (Roth, 1971). A fluorescence reaction is reported to be a more sensitive method of detection than spectrophotometry (Verjat et al., 1999).

For the validation of bioanalytical assays, the Food and Drug Administration (FDA) has detailed experiments to ensure that the assay is able to deliver accurate measurements (Food and Drug Administration, 2001). These experiments allow recording of the sensitivity, specificity, and ability to accurately detect the concentration of unknown substances.

To ensure the aforementioned assays can be used to detect instrument protein contamination, it is important to undertake the experiments detailed by the FDA guidelines to ensure the accuracy and reproducibility of results. The aim of this study was therefore to determine the sensitivity, specificity, and the ability to calculate the protein concentration of common instrument contaminants. The

protein assay was then assessed in an *in situ* study by detecting protein contamination from decontaminated podiatry instruments.

3.2 Results

3.2.1 *Sensitivity and linear range of protein assays*

For the assessment of the linear range and the sensitivity of the protein assays, a 2 mg/ml solution of bovine serum albumin (BSA) and mucin from the bovine submaxillary gland (salivary mucin) were prepared by dissolving 20 mg in 10 ml of RO H_2O. A standard curve of protein concentrations 1, 5,10,20,50,100 µg/ml of protein was prepared in RO H_2O. A blank control of RO H_2O was also included in standard curves. To determine the linear range of each protein assay, BSA concentrations of 50, 100, 250, 500, 1000, 1500, 2000, 2500 µg/ml were sampled. The r^2 value of each graph was calculated using the GraphPad Prism 4 linear regression calculation and the sensitivity was determined using a Kruskal - -Wallis test of all data points in the graph. The sensitivity was determined as the data of the protein concentration that was significantly more than the data of the negative control.

For all assays an increase in BSA protein concentration resulted in a corresponding linear increase in fluorescent units (FU's) for the OPA assay (r^2=0.97) (Figure 3-1), and optical density$_{570}$ OD_{570} for the ninhydrin (r^2=0.98) (Figure 3-2) and BCA assays (r^2=0.99) (Figure 3-3). The OPA and BCA assays had a minimum sensitivity of 5 µg/ml BSA whilst the ninhydrin assay had a minimum sensitivity of 10 µg/ml. An increase in salivary mucin concentration resulted in an increase in FU's for the OPA assay (r^2=0.8) (Figure 3-1) and OD_{570} for the BCA assay (r^2=0.86) (Figure 3-2) and the ninhydrin assay (r^2=0.97) (Figure 3-3), though a smaller straight line slope than was observed in BSA. The limit of detection was calculated as 20 µg/ml for the OPA and ninhydrin assays and 10 µg/ml for the BCA assay. For the BCA assay a linear relationship (r^2=0.92) exists from concentration of 0-1000 µg/ml of protein (Figure 3-4). For the ninhydrin assay, a linear relationship (r^2=0.96) exists to 0-500 µg/ml of protein (Figure 3-5) and the OPA assay has a linear relationship with BSA concentration from 0-2000 µg/ml (r^2=0.97) (Figure 3-6).

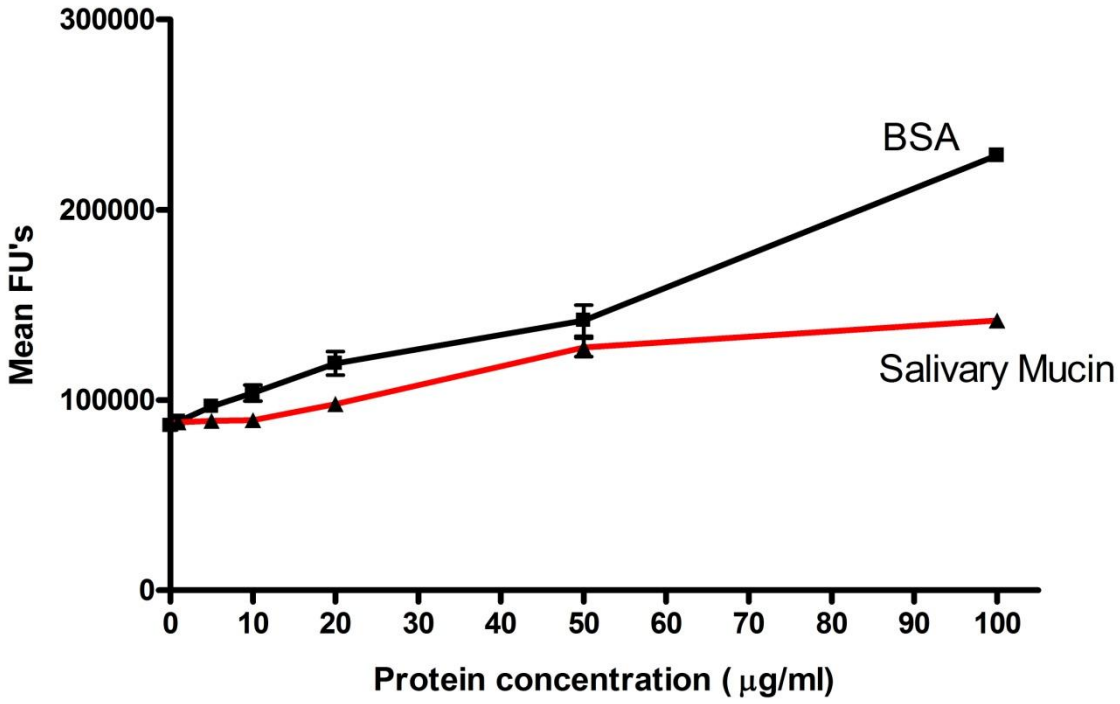


Figure 3-1 BSA and salivary mucin standard curves detected by the OPA assay diluted in RO H_2O. A 2 mg/ml solution of BSA and salivary mucin were prepared by dissolving 20 mg in 10 ml of RO H_2O. A standard curve of protein concentrations 1, 5,10,20,50,100 µg/ml of protein was prepared in RO H_2O. A blank control of RO H_2O was also included in standard curves. The data shown is the mean of 3 readings from 3 experiments ± StEM. A BSA concentration of 5 µg/ml resulted in significantly ($p<0.05$) larger values than the negative control and a salivary mucin concentration of 20 µg/ml resulted in significantly ($p<0.01$) larger values than the negative control. The r^2 value of the slope was calculated as 0.97 for the BSA standard curve and 0.8 for the salivary mucin standard curve.

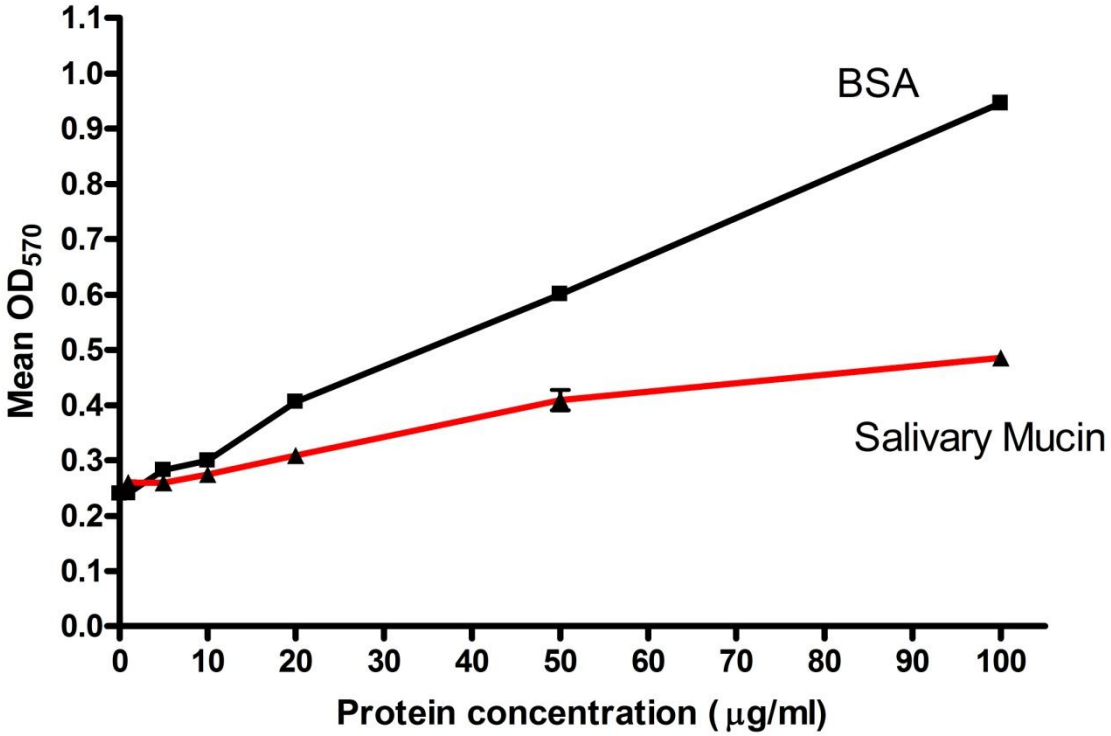


Figure 3-2 BSA and salivary mucin standard curves detected by the BCA assay diluted in RO H_2O. A 2 mg/ml solution of BSA and salivary mucin were prepared by dissolving 20 mg in 10 ml of RO H_2O. A standard curve of protein concentrations 1, 5, 10, 20, 50, 100 µg/ml of protein was prepared in RO H_2O. The data shown is the mean of 3 readings from 3 experiments ± the StEM. A BSA concentration of 5 µg/ml resulted in significantly ($p<0.001$) larger OD_{570} than the negative control and a salivary mucin concentration of 10 µg/ml resulted in significantly ($p<0.01$) larger OD_{570} than the negative control. The r^2 value of the slope was calculated as 0.99 for the BSA standard curve and 0.86 for the salivary mucin standard curve.

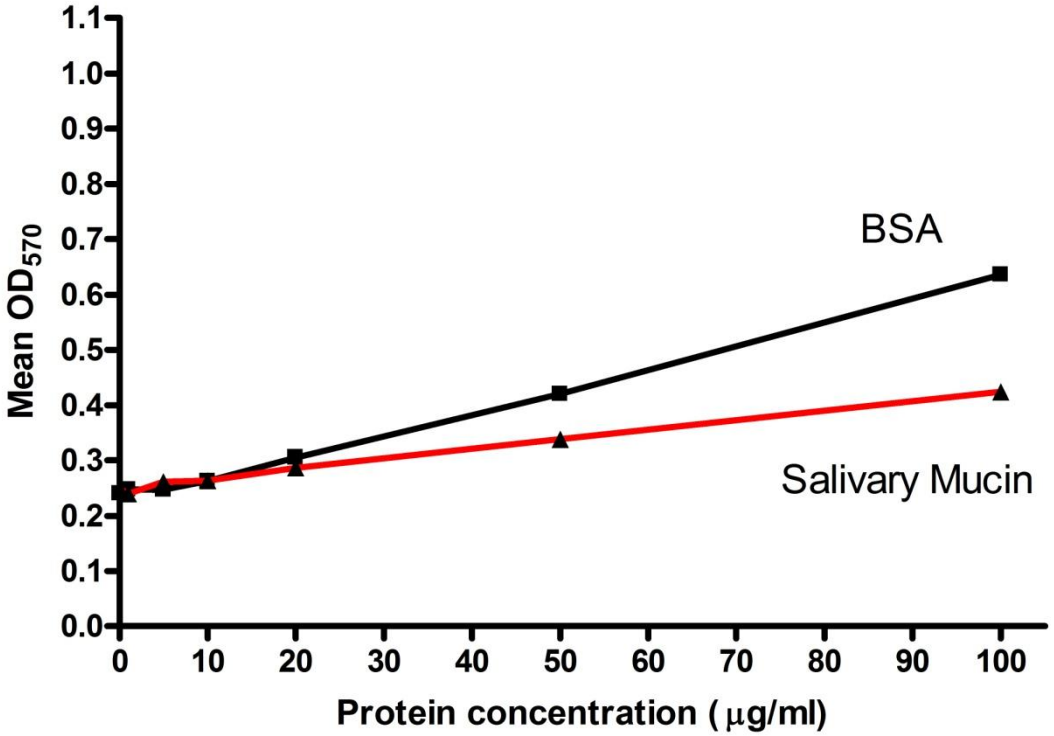


Figure 3-3 BSA and salivary mucin standard curves detected by the ninhydrin assay diluted in RO H_2O. A 2 mg/ml solution of BSA and salivary mucin were prepared by dissolving 20 mg in 10 ml of RO H_2O. A standard curve of protein concentrations 1, 5, 10, 20, 50 and 100 µg/ml of protein was prepared in RO H_2O. The BSA standard curve is indicated with a black line and the salivary mucin standard curve is indicated with a red line. The data shown is the average of 3 readings from 3 experiments ± the StEM. A BSA concentration of 10 µg/ml resulted in significantly ($p<0.01$) larger OD_{570} than the negative control and a salivary mucin concentration of 20 µg/ml resulted in significantly ($p<0.001$) larger OD_{570} than the negative control. The r^2 value was calculated as 0.98 for the BSA standard curve and 0.97 for the salivary mucin standard curve.

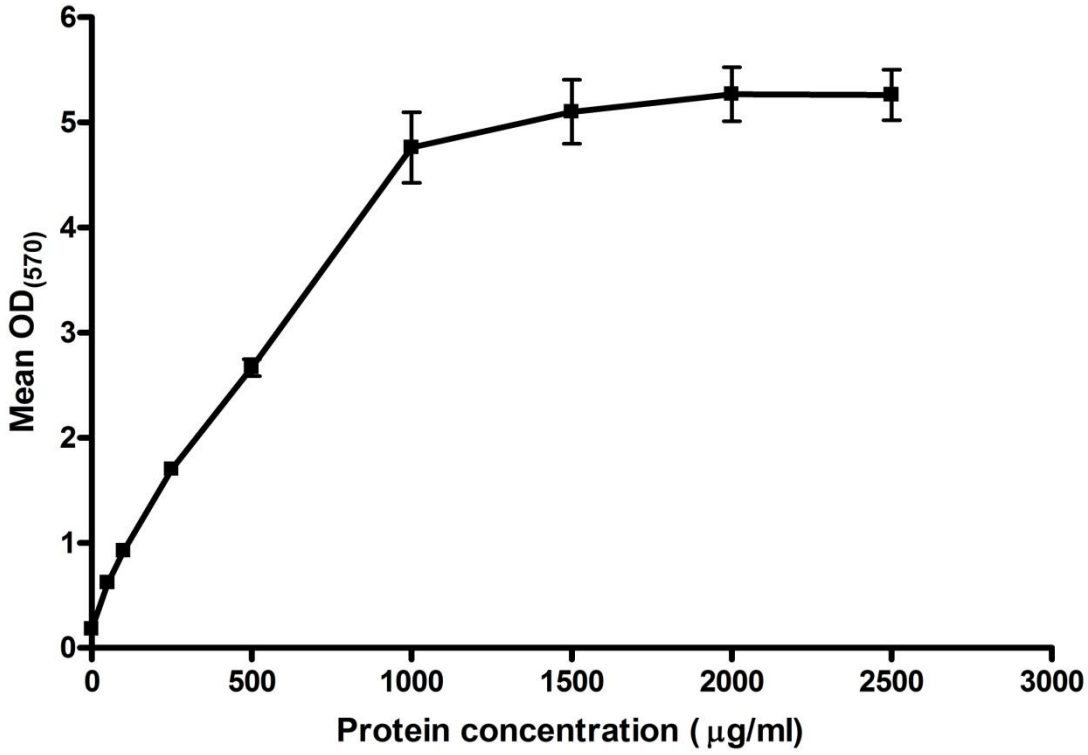


Figure 3-4 Linear range of the BCA assay when detecting BSA. BSA concentrations of 50, 100, 250, 500, 1000, 1500, 2000, 2500 µg/ml were sampled. The data shown is the mean of 3 readings from 3 experiments ± the StEM. A linear relationship (r^2=0.92) was calculated up to 1000 µg/ml.

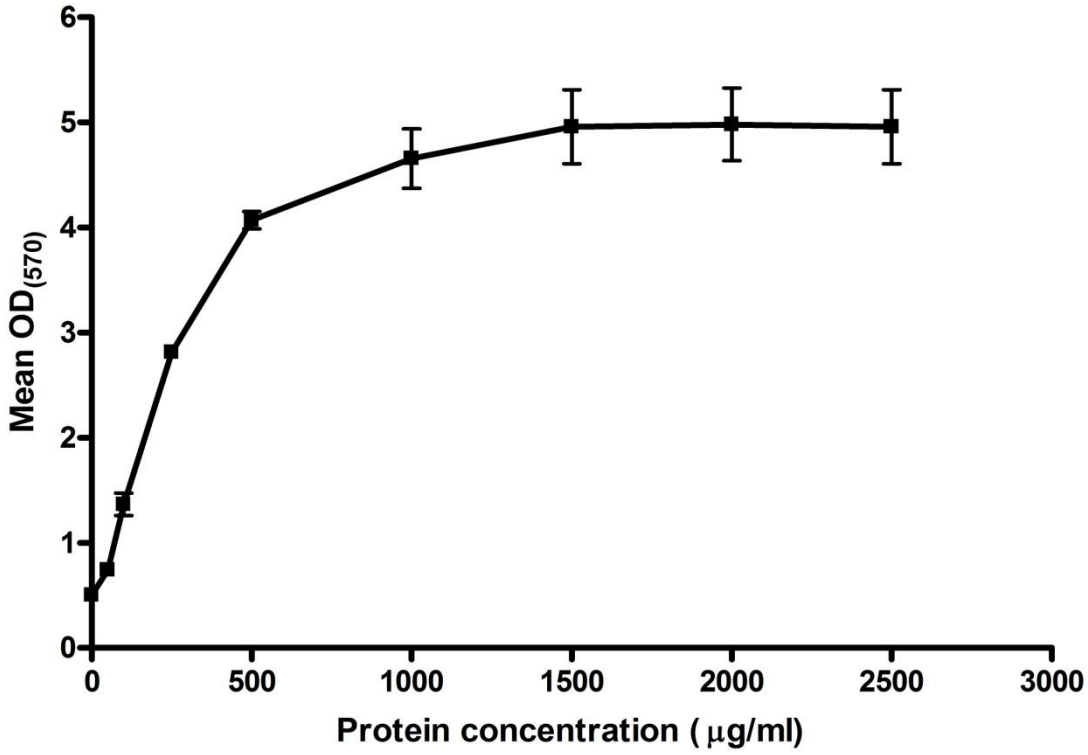


Figure 3-5 Linear range of the ninhydrin assay when detecting BSA. BSA concentrations of 50, 100, 250, 500, 1000, 1500, 2000, 2500 μg/ml were sampled. The data shown is the mean of 3 readings from 3 experiments ± the StEM. A linear relationship (r^2=0.96) was observed up to 500 μg/ml.

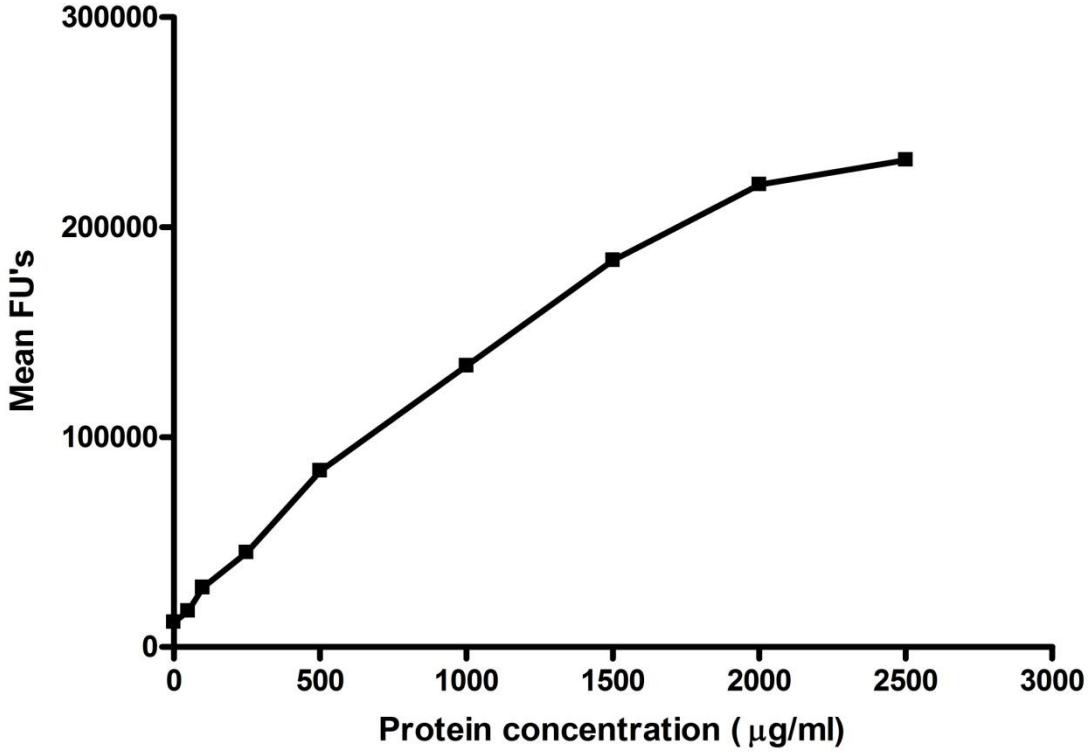


Figure 3-6 Linear range of the OPA assay when detecting BSA. BSA concentrations of 50, 100, 250, 500, 1000, 1500, 2000, 2500 µg/ml were sampled. The data shown is the mean of 3 readings from 3 experiments ± the StEM. A linear relationship (r^2=0.97) was observed up to 2500 µg/ml.

3.2.2 *Protein concentration of unknown biological samples*

Unstimulated saliva was collected separately from 3 male volunteers. Each sample was centrifuged for 15 min at 9971 *g* in a MSE Microcentaur (Sanyo Loughborough, UK) and the supernatant removed. The supernatants were combined and mixed by vortexing. A 1/10 dilution was made in RO H_2O and a sample taken for the ninhydrin, OPA, and BCA assay. Bovine serum, with a reported protein concentration of 45 – 60 mg/ml, was diluted 1/100 in RO H_2O and samples taken for each of the assays. Defibrinated horse blood was diluted 1/1000 in RO H_2O and samples taken for each assay.

Salivary protein concentration was detected as 1.8 mg/ml when sampled with the BCA assay and 1.3 mg/ml with the ninhydrin assay. The OPA assay detected the protein concentration of the saliva samples as 3.9 mg/ml (Table 3-1). For bovine serum, the protein concentration was detected as 53 mg/ml by the OPA assay, 49 mg/ml by the BCA assay and 37 mg/ml for the ninhydrin assay (Table 3-2). For defibrinated horse blood, the OPA assay detected the protein concentration as 121 mg/ml, the BCA assay as 119 mg/ml and the ninhydrin assay as 276 mg/ml (Table 3-3). For unknown BSA concentrations, the OPA assay detected a 20 µg/ml solution was detected as a median of 23.07 µg/ml , a 60 µg/ml solution was detected as 62.64 µg/ml and a 80 µg/ml solution was detected as 80.79 µg/ml (Table 3-4).

Table 3-1 Concentration of salivary protein concentration detected by each protein assay.

Protein assay	Median detected salivary protein concentration (mg/ml)	Range detected salivary protein concentration (mg/ml)
Ninhydrin	1.9	1.5 - 2.2
BCA	1.3	1.2 – 1.4
OPA	3.9	3.6 - 4.2

The data shown is the median and the range of 3 samples from 3 experiments.

Table 3-2 Concentration of serum albumin protein detected by each protein assay.

Protein assay	Median detected serum albumin protein concentration (mg/ml)	Range detected salivary protein concentration (mg/ml)
Ninhydrin	53	49 – 56
BCA	47	44 – 50
OPA	37	36 - 39

The data shown is the median and range of 3 samples from 3 experiments.

Table 3-3 Protein concentration of defibrinated horse blood.

Protein assay	Median total blood concentration (mg/ml)	Range total blood protein concentration (mg /ml)
OPA	121	117– 123
BCA	119	112 – 127
Ninhydrin	276	145 – 216

The data shown is the median and range of 3 samples from 3 experiments.

Table 3-4 Estimated protein concentrations of 3 unknown protein samples.

Known protein concentration (µg/ml)	Median protein concentration detected (µg/ml)	Range protein concentration (µg/ml)
20	23.07	21.96-23.85
60	62.64	62.26 – 64.89
80	80.79	80.12-82.12

The data shown is the median and range of 3 samples from 3 separate solutions made to equal protein concentrations.

3.2.3 *Effect of handpiece lubricating oil and alcohol on protein detection assays*

To determine the effect of lubricating oil and alcohol, the BCA assay was sampled with a 100 µl sample of HP lubricating oil (W & H, St Albans, UK) and 100 µl of HP cleaning solution (W & H, St Albans, UK). A sample of RO H_2O and 100 µg/ml of BSA were also sampled as controls. The experiment was repeated with the ninhydrin assay with 500 µl volumes and the OPA assay with a 20 µl volume of each solution. For the BCA assay and the ninhydrin assay, both W&H manufactured lubricating oil and cleaning solution alone resulted in OD_{570} values significantly ($p<0.001$) more the ROH_2O negative control. For the OPA assay, no significant difference was found between the ROH_2O negative control and the lubricating oil and cleaning solution.

3.2.4 *In situ comparison of automated versus manual cleaning of instruments*

For assessment of the OPA assay at detecting instrument protein, a total of 378 podiatry instruments decontaminated using an LDU and a CDU (Table 2-2) process were assessed for protein contamination. A total of 126 pear burs, 126 Blacks files and 126 Diamond Deb files (Figure 2-2) were collected for the study after single use and randomly allocated into two groups for reprocessing. The first group was subjected to routine cleaning and sterilization by LDU's (Table 2-2) and the second group were subjected to reprocessing by the CDU at Cowlairs (Table 2-2). Individual Blacks and Diamond Deb files were placed in a sterile plastic bag (Seward, UK), whilst each Pear bur was added to a sterile 25 ml Universal tube (Corning, UK). Residual protein was desorbed from each instrument by immersion in a standardised volume of 1% (v/v) SDS (Sigma UK), and for Pear burs only the working end was immersed. Each instrument was subjected to sonication at 35 kHz for 30 min in an ultrasonic bath (Thermofisher Fisherbrand® 11021) (Smith et al., 2005) and the protein desorbed measured using the OPA assay (Section 2.3.1.3) compared to a BSA standard curve (Section 3.2.1).

A total of 58/63 Pear burs, 48/63 Blacks files and 31/63 Diamond Deb files reprocessed by CDU contained greater than 5 µg/instrument of detectable protein. Protein was also detected in 62/63 Pear burs, 53/63 Black files, and 56/63 Diamond Deb files reprocessed by LDU (Figure 3-7). Instruments reprocessed by the CDU (median 21 µg/instrument range <5 -5705 µg/instrument) had significantly less residual protein than instruments reprocessed by the LDU (median 117 µg/instrument range <5 – 6344 µg/instrument) when all three instruments were grouped ($p<0.001$ (Figure 3-7).

For individual instruments, the median quantity of protein detected on Pear burs (Figure 3-8) reprocessed by CDU was significantly lower (median 11 µg/instrument range <5-161.7 µg/instrument) than those by LDU (median 77 µg/instrument, range <5–1403 µg/instrument $p<0.001$). The median quantity of protein detected on Blacks files (Figure 3-9) reprocessed by CDU (median 64.52 µg/instrument, range <5-1113 µg/instrument) exhibited no significant difference

compared to protein detected on Blacks files by LDU (median 50.81 µg/instrument, range <5-633.5 µg/instrument). The median quantity of protein detected on Diamond Deb files (Figure 3-10) reprocessed by CDU was significantly lower (median <5 µg/instrument, range <5 – 5705 µg/instrument) than Diamond deb files reprocessed by LDU (median 711.8 µg/instrument, range <5 – 6344 µg/instrument) ($p<0.05$). However, residual protein was still detected from these instruments, as the mean of these was 512 µg/instrument for CDU reprocessing compared to 1159 µg/instrument for LDU reprocessing, indicating that a small proportion of CDU samples contained elevated levels of residual protein.

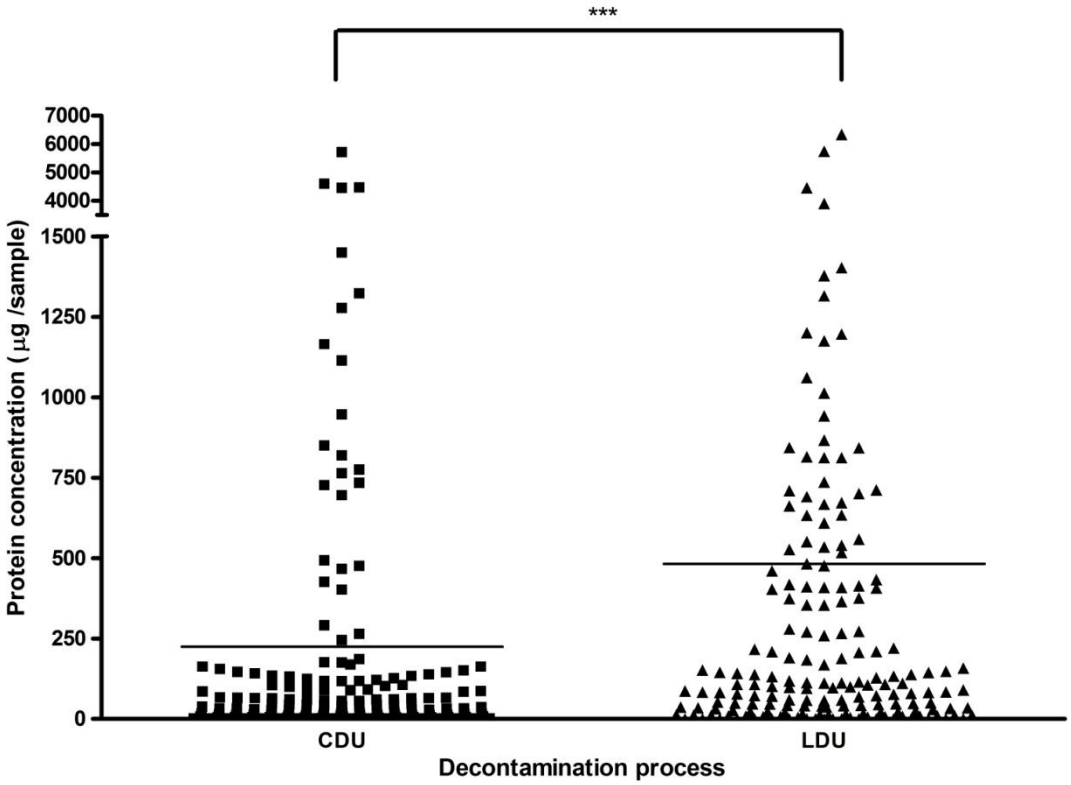


Figure 3-7 Residual protein isolated from all instruments after reprocessing by both methods. Protein was desorbed from all instruments by immersion in 1% (v/v) SDS and sampled using the OPA assay.. The distribution of data was confirmed using a Kolmogorov Smirnov test, and data was compared using a Mann-Whtiney U test with the significance determined using a 2-tailed Monte Carlo estimation. Significantly less protein is recovered from instruments reprocessed using the CDU method (***= $p<0.001$) compared to instruments reprocessed using the LDU (Table 2.2). The mean of datasets is represented by a horizontal line.

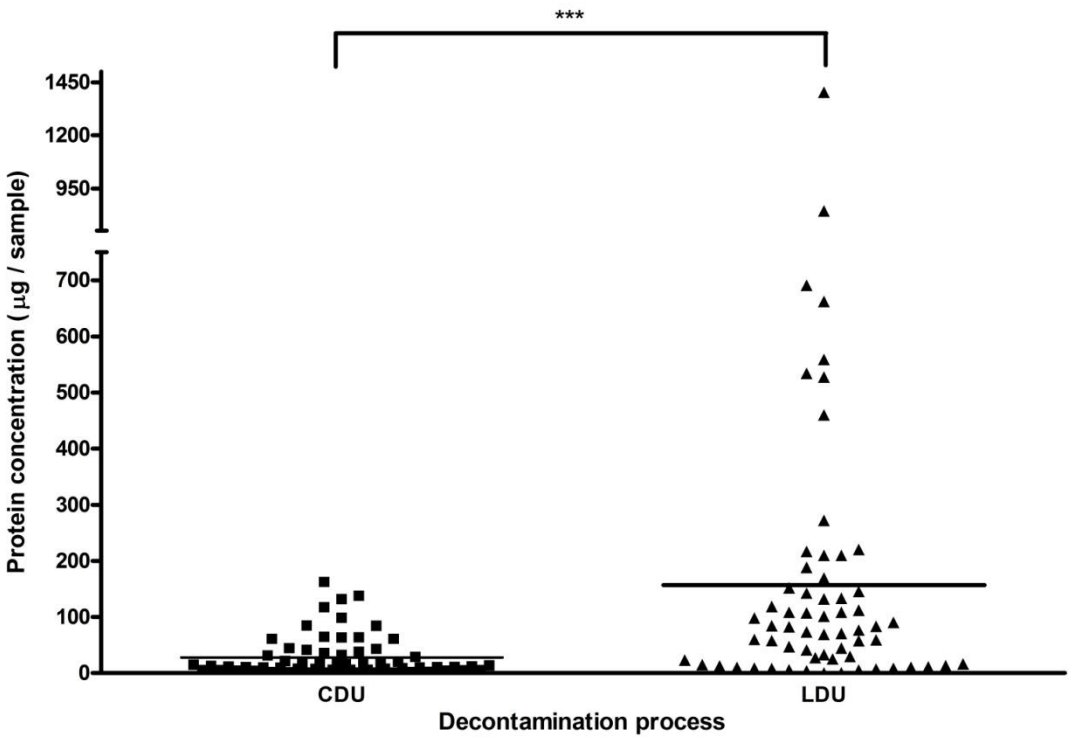


Figure 3-8 Total residual protein recovered from individual Pear burs reprocessed by both methods. Protein was desorbed from all instruments by immersion in 1% (v/v) SDS and sampled using the OPA assay. The distribution of data was confirmed using a Kolmogorov Smirnov test, and data was compared using a Mann-Whtiney U test with the significance determined using a 2 tailed Monte Carlo estimation. Significantly less protein is recovered from instruments reprocessed using the CDU method (***= $p<0.001$) compared to instruments reprocessed using the LDU (Table 2.2). The mean of datasets is represented by a horizontal line.

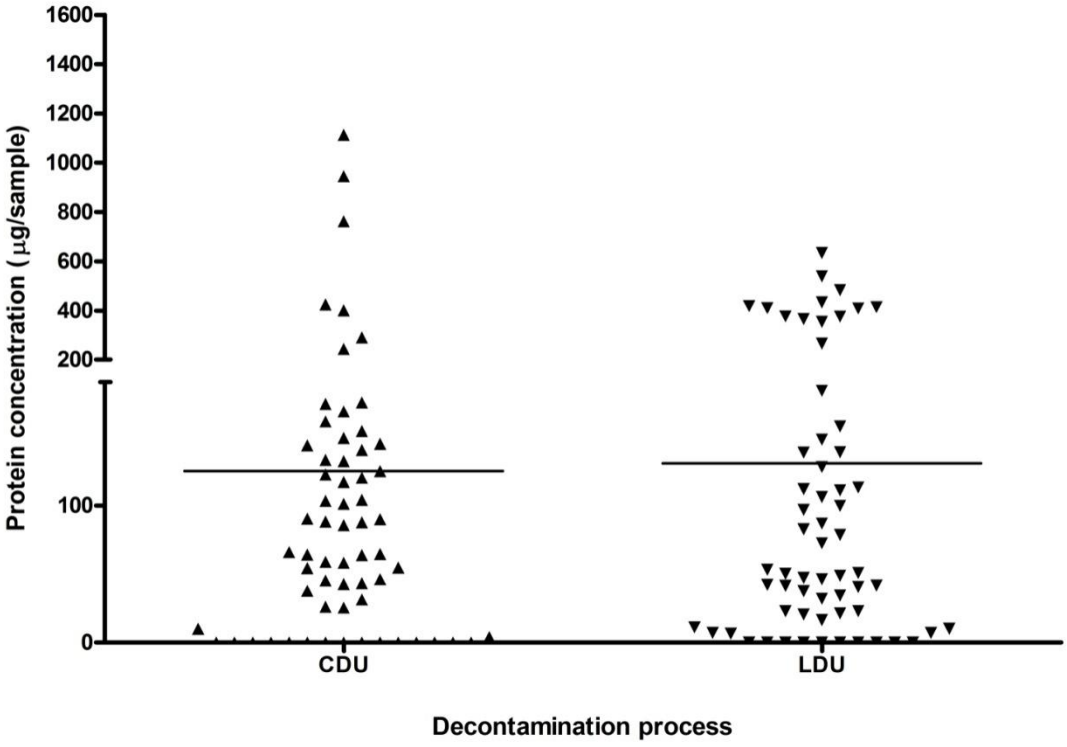


Figure 3-9 Total residual protein recovered from individual Blacks files reprocessed by both methods. Protein was desorbed from all instruments by immersion in 1% (v/v) SDS and sampled using the OPA assay. The distribution of data was confirmed using a Kolmogorov Smirnov test, and data was compared using a Mann-Whitney U test with the significance determined using a 2 tailed Monte Carlo estimation. No significant difference was found on protein recovered from instruments reprocessed using the CDU method and the LDU (Table 2.2). The mean of datasets is represented by a horizontal line.

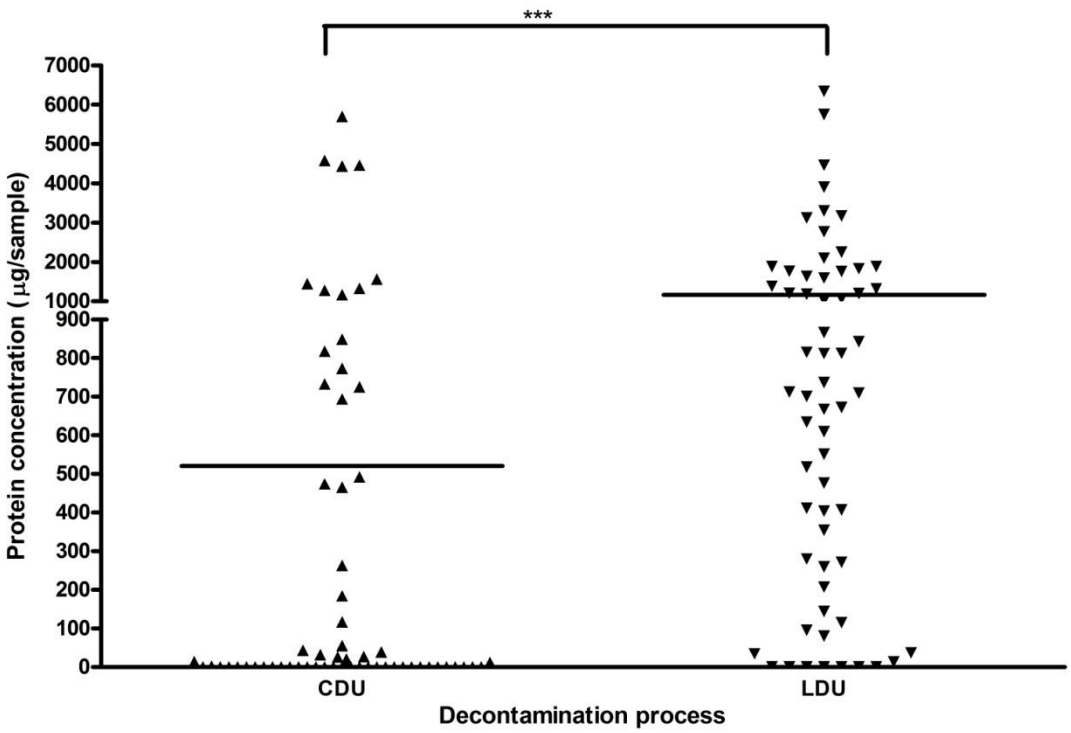


Figure 3-10 Total residual protein recovered from individual Diamond deb files reprocessed by both methods Protein was desorbed from all instruments by immersion in 1% (v/v) SDS and sampled using the OPA assay. The distribution of data was confirmed using a Kolmogorov Smirnov test, and data was compared using a Mann-Whitney U test with the significance determined using a 2-tailed Monte Carlo estimation. Significantly more protein was detected on instruments reprocessed using the LDU process (***=$p<0.001$) than instruments reprocessed using the CDU process (Table 2.2). The mean of datasets is represented by a horizontal line.

3.3 **Discussion**

Cleaning validation of washer disinfectors rely on the detection of protein using the ninhydrin, the BCA and the OPA assays. This standard defines an acceptable cleaning level as below the detection limit of one of three assays, which are stated as 2 mg/m^2 for the ninhydrin assay, 30 – 50 µg for BCA, and 0.003 µmol of OPA sensitive amino groups for the OPA assay (BS-EN-ISO-15883, 2006). The validation of protein assays can be accomplished using the FDA bioanalytical guidelines, which detailed the experiments performed in this study, i.e. assessing the sensitivity. The range of detection of the assays, the ability to detecting common dental instrument protein contamination, and the interference by common HP maintenance solutions that may be present during HP sampling (Food and Drug Administration, 2001).

BSA is commonly used as protein standards to compare unknown protein samples (Smith et al., 2005), especially for detecting blood contamination (Doumas, 1975) which is a common contaminant of the instruments sampled and is present in all test soils described by the BS-EN ISO -15883:2006.

The BCA and the OPA assays showed a sensitivity of 5 µg/ml of protein and the ninhydrin assay had a sensitivity of 10 µg/ml. This was the minimum BSA concentration that did not result in an OD significantly greater than the negative control. Based on the difference in sample volumes required for each reaction, the OPA assay can detect 100 ng of protein, the BCA assay can detect a minimum of 0.5 µg of protein and the ninhydrin assay can detect a minimum of 5 µg of protein. The sensitivity of the BCA assay was identical to that stated by the manufacturer (Pierce Biotechnology, 2009) and in previous studies (Smith et al., 1985). The ninhydrin assay has a reported sensitivity of 9.75 µg when used as a kit relying on swabbing and visual analysis of the swabs (Lipscomb et al., 2006a). This figure is similar to the sensitivity observed in this study. The OPA assay has a reported sensitivity of 1.2 ng of protein (Hammer and Nagel, 1986) and 3.6 µg/ml (Verjat et al., 1999) depending on the method of protein preparation and the size of sample. The figure obtained by Verjat *et al*. (1999) was similar to that observed in this study.

The range of protein detected by each protein assay impacts the concentrations of protein that can be detected by each assay. The OPA assay had the longest linear range of 0 – 2500 µg being detected. Since this assay relies on relative FU's for protein measurement, it is possible to adjust the protein standards to appropriate concentrations when larger protein concentrations are expected. The range of the BCA assay in this study was detected as 0 - 1000 µg/ml, which is lower than that stated by the manufacturer, 0 - 2000 µg/ml. This study altered the method by halving the manufacturer total recommended reagent which had no impact in sensitivity of the assay, but may have impacted on the reaction products available. The linear range of the ninhydrin assay was the smallest of the assays sampled.

The ability of each assay to detect salivary mucin, a common salivary protein was also assessed (Liu et al., 1998). Each assay underestimated the concentration of salivary mucin shown by the change of sensitivity and line slope for salivary mucin standard curves. This change in sensitivity may have implications for detecting salivary protein contamination on instruments. Salivary mucin is the main constituent of mucins and undergoes post-translational glycosylation, making any mucin resistant to proteolysis and therefore may also inhibit the reaction with the protein assays. The BCA and the ninhydrin assay can detect salivary mucin at a smaller concentration of 10 µg/ml than the OPA assay, which detects salivary mucin at 20 µg/ml.

The ability of the protein assays to detect common instrument contaminants was assessed. The protein concentration of each sample was unknown for the purposes of the study. For the determination of protein concentration of human saliva, the OPA assay detects a higher level of protein concentration than the BCA and the ninhydrin assay. Human salivary protein concentration has been calculated as 1.8 mg/ml (Lamanda et al., 2007), 1.07 mg/ml (Narhi et al., 1994), and 6.68 mg/ml (Agha-Hosseini et al., 2006). Each of the findings was assessed by the Bradford assay, based on the binding of Coomassie dye to the amine groups of the protein (Bradford, 1976). Salivary protein concentration has many variables that would have an effect on salivary protein studies including age, sex, saliva stimulation, diet, and health. The findings from this study from the ninhydrin and the BCA assay are similar to those reported by Lamanda

(2007), although the OPA assay calculates the same salivary samples as having double the protein concentration. This may be due to cross reactivity with a salivary component and this phenomenom may lead to an over estimation of salivary protein concentration by the OPA assay.

The ability of each assay to detect blood protein and serum protein concentration was assessed. Blood contamination can also be detected using the Kastle-Meyer test and visual contamination (Lowe et al., 2002, Zuhlsdorf and Martiny, 2005) though these methods do not allow quantitative measurement of the blood proteins. The BS-EN-ISO-15883:2006 standard relies on visual contamination and the detection of any blood protein using the 3 protein assays. The OPA and the BCA assay both detected a similar protein concentration when defibrinated horse blood though the ninhydrin assay estimates the blood protein contamination as double that of the other assays. The haemoglobin in blood is known to interfere with the ninhydrin assay, which may be due to the presence of interfering substances in the blood solution (Burzynski, 1969). The protein concentration of bovine serum is reported between 45 to 75 mg/ml as detected by the Biuret reaction by the manufacturer. All protein assays detect a protein concentration within these limits and the BCA and OPA assay both detect similar protein concentrations. For the unknown BSA concentrations only the OPA assay was assessed due to its sole suitability for assessing HP contamination. All the concentrations were detected within the 15% of the known protein concentration further validating the suitability of the OPA assay for HP protein analysis.

Lubricating oil and cleaning alcohol, both essential parts of HP maintenance, both react when in contact with the BCA and the ninhydrin assay. This implies that each solution contains a chemical that cross reacts with both of the assays. The BCA assay will show a positive reaction in the presence of any copper chelating agent such as dimethyl sulfoxide (DMSO) or any agent that changes the pH of the BCA reagent (Smith et al., 1985). The ninhydrin assay can also react with aldehydes, ketones, keto acids, and monosaccharide's (Schilling et al., 1963), which result in a false positive reaction. The lubricating oils specific composition is not known, however, the material safety data sheet (MSDS) indicates the presence of ester oils that contain amino acids that can react with

the ninhydrin assay. The cross reaction with these products with the BCA and the ninhydrin assay has implications for the use of these assays to detect protein contamination in a HP. Lubricating oil and cleaning alcohol are part of routine handpiece reprocessing and the positive reactions may falsely indicate protein contamination. No such cross reactivity was found with the OPA assay, indicating its sole suitability for assessing handpiece protein contamination.

The OPA assay was subsequently used to detect protein contamination of 3 different podiatry instruments, pear burs, Blacks files and diamond deb files (Figure 2-2). Each instrument has a complex surface topography and each is exposed to skin and protein during routine use. This makes them ideal instruments for the validation of the OPA assay to detect protein contamination from decontaminated instruments and validate 2 currently used podiatry decontamination processes.

When all podiatry instruments were grouped, the CDU instruments were found to contain significantly less residual protein than an identically sized group of instruments reprocessed by the LDU. The reason for the difference in cleaning efficacies between the CDU and the LDU are multifactorial and include a more robust validation process for the automated washer disinfectors in use at the CDU. Other factors include an increased cleaning process time in the CDU (11 min- CDU compared to 6 min - LDU), different cleaning chemistries used, the differences in form of energy used in cleaning processes, and different temperatures used during the wash stage.

Similar patterns of cleaning efficacy were observed within each group of instruments with the exception of Blacks files, which may be due to the smaller ridged surface area compared to the more complex surface topography associated with the other instruments. This characteristic has been associated with increased retention of contamination by surface analysis of endodontic files which also have a ridged surface topography (Smith et al., 2002).

If using the BS-EN-ISO-15883 standard as a threshold for cleanliness for reprocessed instruments, a total of 68/189 instruments reprocessed by CDU and 19/189 instruments reprocessed by the LDU would be deemed to be clean. The

number of clean instruments may drop considerably if more sensitive analytical procedures were employed.

The data reported herein highlights the superiority of the CDU process in terms of cleaning efficacy at reprocessing more complex instruments and shows the suitability of the OPA assay at detecting protein contamination isolated from instruments. Previous studies have focused on the efficacy of CDU reprocessing by assaying a range of surgical instruments containing residual protein that was detected after reprocessing (Murdoch et al., 2006, Smith et al., 2005). The protein content of different surgical instruments, including metzenbaum scissors and forceps, ranged from 163 to 756 µg, which is similar to that reported herein (Murdoch et al., 2006, Baxter et al., 2006). Similarly, a study on reprocessed dental endodontic files, which have a complex surface topography, showed a range of protein from 0.2 to 63.2 µg, similar to those levels observed on the Pear burs (Smith et al., 2005).

In order to improve validation of instrument reprocessing from visual inspection and published standards, techniques with greater quantitative sensitivity have emerged. Examples include a fluorescent microscopy technique involving visualisation of protein by SYPRO ruby staining capable of detecting 85 pg of protein on a surface area of $1mm^2$ which is significantly lower than the sensitivity of 5 µg/instrument reported in this study (Lipscomb et al., 2006b). A standard for cleanliness when considering protein contamination should be dependent on the procedures undertaken by the instrument. The total protein recovered from the podiatry instruments would be equivalent to a large number of prion infectious units (Lipscomb et al., 2006a).

Each protein assay has been assessed for its suitability at detecting common dental contaminants. While each has its short comings, each assay is a cheap method that can be reproduced in a sterile services department. The OPA method validated in this study requires a small protein sample, a short preparation time, identical sensitivity to commercial kits and has a longer linear range than both the BCA commercial kit and the ninhydrin solution. The ninhydrin and the BCA assay both failed the FDA bioanalytical standards due to false positive reactions of the lubricating oil and alcohol cleaning solution of the

dental HP. Constant work is needed to further validate and improve protein detection tests to ensure the validated cleaning of instruments and to determine cross contamination risks of all invasive surgical instruments.

4 Chapter 4: Quantitative and Qualitative Analysis of Microbial Contamination of Dental Handpieces

4.1 Introduction

During routine use, HP's are contaminated from the external environment through a variety of means, including negative pressure created by the deceleration of the turbine when the air supply is stopped (Matsuyama et al., 1997) and from the dental unit water line that provides cooling water to the burs (Atlas et al., 1995). The HP contaminants internalised may be expelled during subsequent re-use and may provide a cross-infection risk if decontamination procedures are not undertaken. The reprocessing of HP's is challenging due to the complex internal structures, narrow lumens and lack of disassembly after use (Lewis and Boe, 1992).

The oral cavity is home to a large number of bacteria which grow on the non shedding tooth surface in the form of a biofilm (Paster et al., 2001), and the *in-vivo* contamination of dental instruments and HP's with bacteria has been reported since the 1800's and reviewed specifically for HP's as early as 1924 (Miller, 1891, Appleton Jr and L, 1924). Since these early studies HP's have been developed with more diverse uses, and manufacturers have attempted to implement design features to prevent the internalisation of contamination (Hu et al., 2007b). New decontamination technologies such as washer disinfectors and vacuum sterilizers have also provided automated, reproducible cleaning, and sterilization for the internal HP parts (Andersen et al., 1999). Despite these advances, bacteria have been reported to be isolated from HP's prior to decontamination in an *in vivo* study, conducted as recently as 2007 (Herd et al., 2007). New techniques for the isolation and identification of bacteria have also been developed that increase the sensitivity of recovery and can identify bacteria that are non-culturable (Bjerkan et al., 2009).

It is important to determine the typical microbial contamination of routinely used HP's before and after the cleaning and sterilization process to understand the current biological and chemical challenges to these processes. Knowledge of the HP contaminants prior to decontamination will inform the development of cleaning processes and chemicals whilst any contaminants remaining after decontamination will inform on risk assessment and cross contamination issues.

The aim of this study was to undertake a quantitative and qualitative analysis of the bacterial contamination of the internal parts of used unprocessed and decontaminated air turbine, low speed, and surgical HP's from a dental hospital and a HP repair facility utilising culture and culture independent techniques.

4.2 Results

4.2.1 *Relationship between bacterial colony forming units and optical density*

To determine the relationship between CFU and OD, *S. epidermidis* and *P. aeruginosa* type strains (Table 2-5) were cultured as detailed in section 2.2.3. Each culture was centrifuged at 2215*g* for 10 min in a MSE Centaur 1 centrifuge (Sanyo Loughborough, UK), the supernatant was removed and the cells resuspended in 10ml of sterile PBS. The OD of the resuspended culture was determined using a Colorimeter model 45 spectrophotometer (Fisher Scientific, Longborough UK) and the culture diluted to OD's of 0.2, 0.25 and 0.3. Each OD sample was given 3x 1/10 serial dilutions in sterile PBS and 3 x 10 µl samples of each dilution were plated onto a BA plate and incubated aerobically overnight at 37°C.

An increase in OD resulted in an increase in CFU for *S. epidermidis* (Figure 4-1 and *P. aeruginosa* (Figure 4-2). An OD of 0.4 was equivalent to $1x10^8$ cfu for *S. epidermidis* and 0.2 for *P. aeruginosa*.

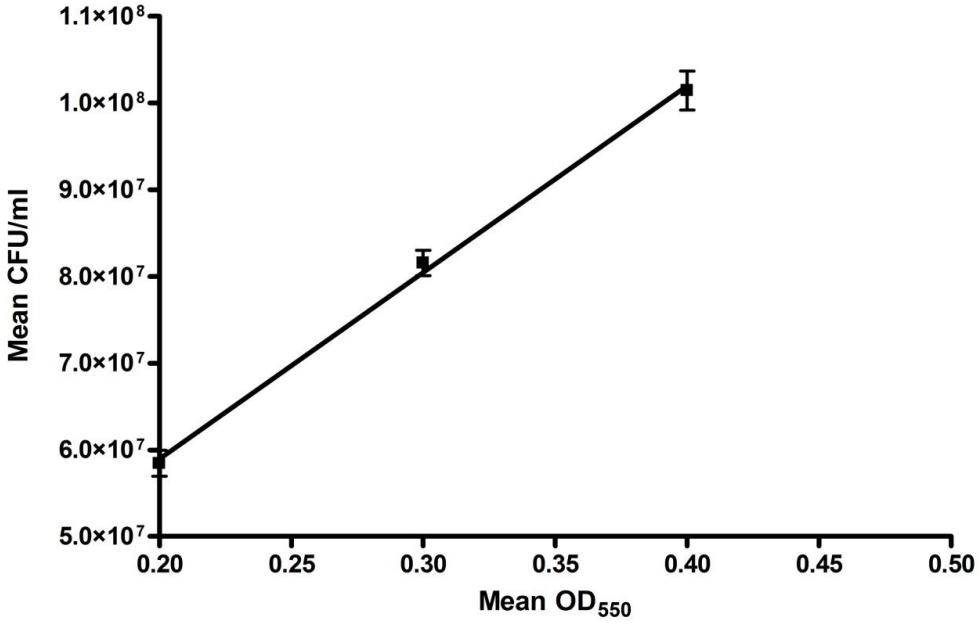


Figure 4-1 Relationship between *S.epidermidis* CFU/ml and OD. Cultures of *S. epidermidis* were diluted to appropriate OD values, diluted and cultured to determine the CFU/ml of each OD. An increase in OD results in an increase in CFU/ml.

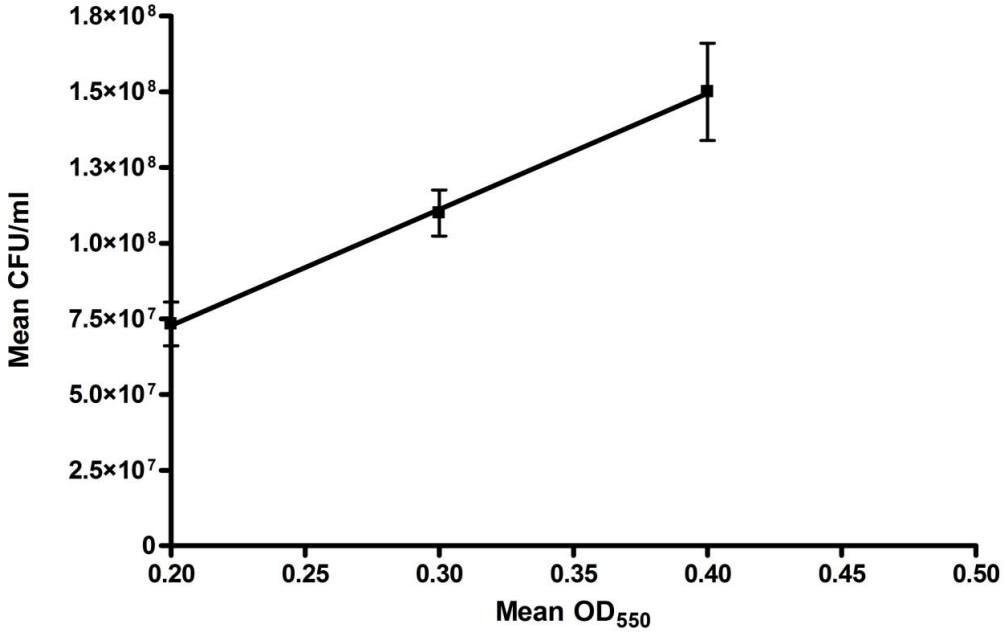


Figure 4-2 Relationship between *P. aeruginosa* CFU/ml and OD. Cultures of *P. aeruginosa* were diluted to appropriate OD values, diluted and cultured to determine the CFU/ml of each OD. An increase in OD results in an increase in CFU/ml.

4.2.2 *Minimum inhibitory concentration of handpiece lubricating oil and cleaning solution*

To determine the minimum inhibitory concentration (MIC) of HP lubricating oil and cleaning solutions, *S. epidermidis, S. aureus, P. aeruginosa, S. mutans, S. salivarius, S. sanguinis,* and *C. albicans* type strains (Table 2-5) were cultured as detailed in section 2.2.3. Each culture was centrifuged for 10 min at 2215 *g* in a MSE Centaur 1 centrifuge (Sanyo Loughborough, UK) and resuspended in 10ml of sterile PBS. Each microbial culture was diluted to equivalent of 1 $x10^5$ CFU/ml (Section 4.2.1). The *C. albicans* culture was given 2x 1/10 serial dilutions and 8 µl of the 1/100 dilution was added to a hemocytometer for cell counting. The *C. albicans* culture was then diluted to a concentration of 1 $x10^4$ CFU/ml. A total of 100µl of MH broth for sampling bacteria or RPMI broth for sampling *C. albicans* was added to each well of 4 rows and columns 2-12 of a clear flat bottomed 96 well plate. A 100 µl sample of lubricating oil (W&H, Burmoos Austria) or cleaning solution (W&H, Burmoos Austria) was added to column 1 of the 96 well plate and given 9x 1/2 serial dilutions by adding 100 µl of the oil or alcohol to the 100 µl of broth in column 2 and repeating up to column 10. A 100 µl sample of bacteria or *C. albicans* was added to four rows of columns 1-10 and column 12 as a positive control. Each plate was incubated overnight aerobically at 37 °C and the growth of each well measured using a Sunrise™ plate reader (Tecan Mannedorf, Swtizerland) at OD_{670}.

Growth was observed for all strains when exposed to all concentrations of lubricating oil. Cleaning solution had an inhibitory growth effect on all strains tested (Table 4-1).

Table 4-1 MIC of HP maintenance solutions.

Organism	Lubricating oil MIC (% Solution)	HP Cleaning Solution MIC (% Solution)
S. epidermidis	No Inhibition	6.25
S. aureus	No Inhibition	6.25
P. aeruginosa	No Inhibition	3.125
C. albicans	No Inhibition	3.125
S. mutans	No Inhbition	6.25

Data shown is representative of 3 replicates from 3 experiments.

4.2.3 *Sensitivity of extraction methods*

To determine the sensitivity of extraction methods *S. epidermidis* of concentrations $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ CFU/ml were inoculated on the surface of decontaminated SSS (Section 2.5.1.1). Sterile PBS was added to SSS's as negative controls. Each SSS was dried for 2h in a laminar flow cabinet. A total of 3 SSS's were sampled by swabbing and 3 SSS's of each concentration sampled by sonication. For sonication, each SSS was added to a sterile Bijoux tube (Sterilin, Caeriphilly UK) and each tube was inserted into a Fisherbrand® 11021 sonic bath (Fisher Scientific, Loughborough UK) filled with ROH_2O and sonicated for 5 min at 35 kHz (Smith et al., 2005). For swabbing, sterile swabs were soaked in sterile PBS and the SSS surfaces were swabbed for 5 sec which was the length of time needed to cover the surface 3 times. Each swab head was then immersed in 1 ml of sterile PBS and vortexed for 5 min. The eluents were then given 3 x 1/10 serial dilutions and a 10 µl sample of each dilution was added to a BA plate and the plates incubated overnight at 37°C under 5% CO_2 (Binder, Tuttlingen Germany).

No growth was detected on the negative control samples. Swabbing of SSS's resulted in 67% less recovery of *S. epidermidis* than the sonication technique (Table 4-2). For swabbing, no bacteria were recovered at a starting concentration of $x10^4$ CFU's and for sonication no bacteria were recovered from a starting concentration of $x10^2$ CFU's.

Table 4-2 Percentage recovery of each extraction method at recovering *S. epidermidis*.

Extraction Method	Starting bacterial concentration (CFU/ml)	Bacteria recovered by swabbing (CFU/ml)	% Recovery swabbing	Bacteria recovered by sonication (CFU/ml)	% Recovery sonication
Swabbing	$4.2x10^6$	$1.5x10^5$	4.6	$3.1x10^6$	71.4
	$4.1x10^5$	50	0.02	$2.6x10^5$	63
	$4.1x10^4$	0	0	$2.4x10^4$	58
	$3.8x10^3$	0	0	$2.5x10^3$	65
	$4x10^2$	0	0	0	0
	$4x10^1$	0	0	0	0
	0	0	0	0	0

Data shown is the mean of 9 samples from 3 experiments.

4.2.4 *Microbial contamination of Glasgow Dental Hospital dental handpieces*

No bacteria were isolated from the negative controls. Before decontamination, bacteria were isolated from a total of 38/40 turbines (median 200 CFU/instrument, range 0 – $1.9x10^3$ CFU/instrument), 37/40 spray channels (median 400 CFU/instrument, range 0 – $1x10^3$ CFU/instrument), and 18/20 surgical gears (median $1x10^3$ CFU/instrument, range 0 – $3.7x10^4$ CFU/instrument) (Figure 4-3). The majority of organisms isolated from each location were CONS with oral streptococci, *Staphylococcus aureus*, Gram positive bacilli, *Pseudomonas spp*. and *Propionibacterium acnes* (Figure 4-4, Figure 4-5, Figure 4-6, Table 4-3).). A total of 11 *P. acnes* isolates from GDH HP's were identified by typing (Table 4-4). After decontamination, bacteria were isolated from 5 spray channels (median 30 CFU/instrument, range of 0 – $1.5x10^2$ CFU/instrument) (Figure 4-3). No bacteria were isolated from decontaminated turbines and surgical gears.

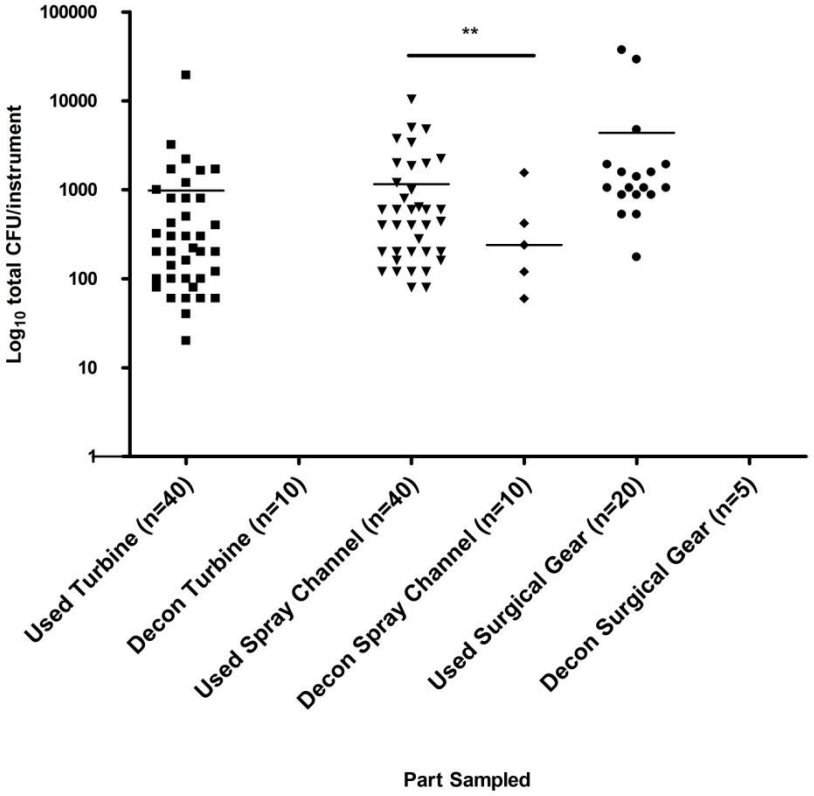


Figure 4-3 Number of bacteria isolated from each HP part. Internal HP parts were sampled for bacteria before and after decontamination including sterilization. Each part was cultured for aerobic and facultative anaerobic isolates. Before decontamination, surgical HP gears contained the most bacteria with a median of $1x10^3$ CFU compared to a median of 200 and 400 CFU for used unprocessed turbines and spray channels respectively. After decontamination (Table 2.2), no bacteria were isolated in turbines and surgical gears whilst a median of 30 CFU of bacteria was isolated from decontaminated spray channels which was a significant reduction (**$p<0.01$). The mean of datasets is represented by a horizontal line.

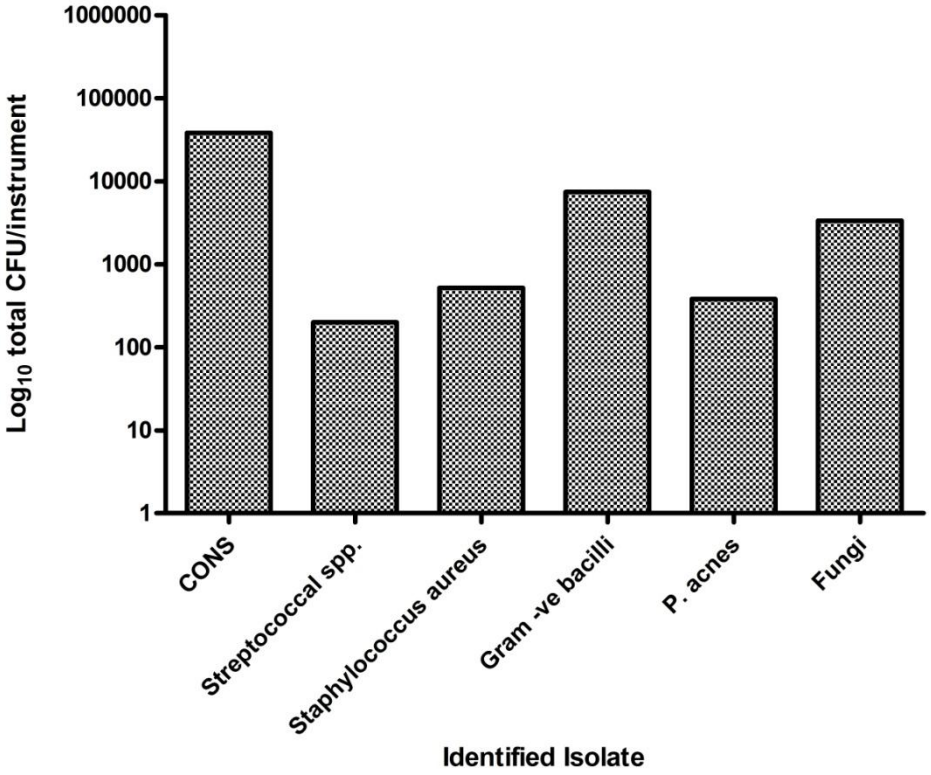


Figure 4-4 Total number of isolates from high speed turbines. Bacteria were identified using diagnostic microbiology. All isolates were from used, unprocessed samples. The majority of bacteria isolated were CONS with a total of $3.8x10^4$ isolates followed by Gram negative bacilli with a total of $7.5x10^3$ isolates and unidentified fungi with a total of $3.3x10^3$ isolates from all turbines sampled. *S. aureus*, oral *Streptococci* and *P. acnes* were isolated in smaller numbers with a total of $5x10^2$ *S. aureus,* $2x10^2$ oral *Streptococci*, and $3.8x10^2$ *P. acnes.*

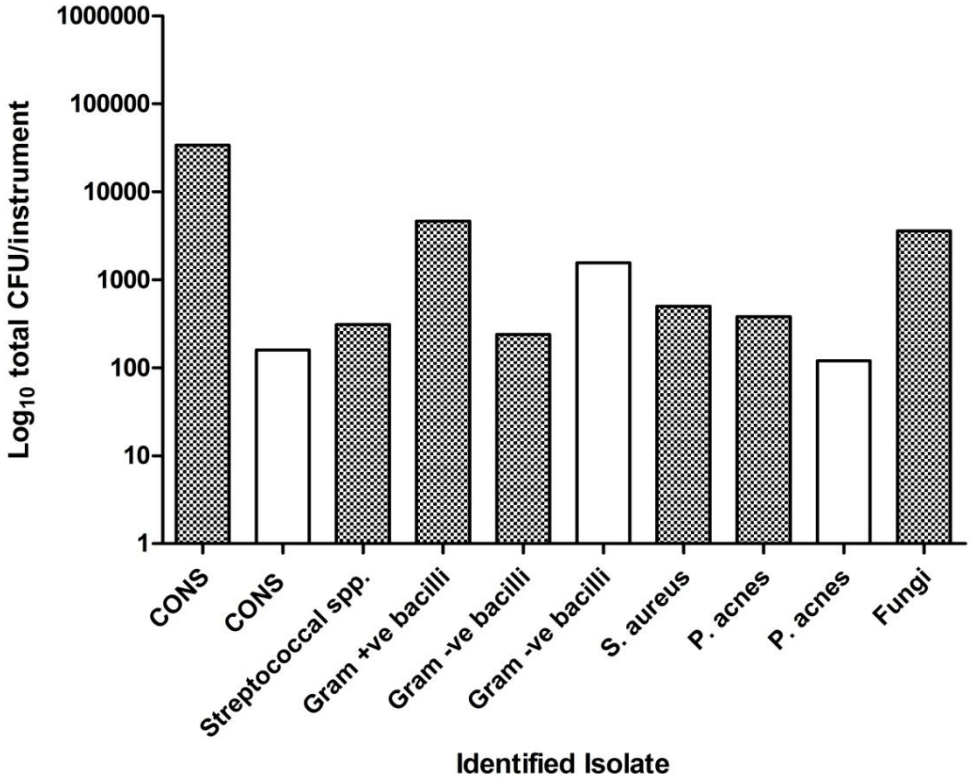


Figure 4-5 Total number of isolates from low speed spray channels. Bacteria were identified using diagnostic microbiology techniques. Isolates from used, unprocessed handpieces are highlighted by gray bars and isolates from decontaminated HP's are represented by white coloured bars. Before decontamination, the majority of bacteria isolated were CONS with a total of 3 $x10^4$ CFU and unidentified fungi with a total of $3.6x10^3$ CFU from all spray channels. After decontamination (Table 2.2), CONS were reduced to $1x10^2$ CFU whilst no reduction in Gram negative bacilli or *P. acnes* was observed.

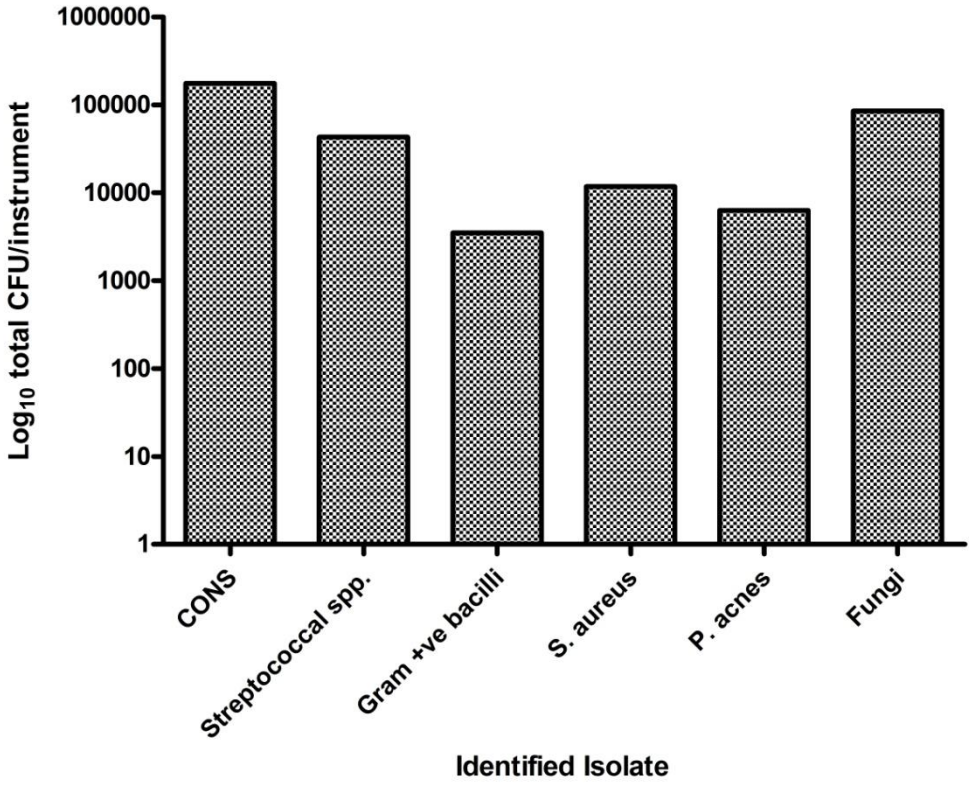


Figure 4-6 Total number of isolates from surgical gears. Bacteria isolated were identified using diagnostic microbiology techniques. All isolates were from used, unprocessed HP's. The majority of bacteria isolated were coagulase negative staphylococci (CONS) with a total of $1.7x10^5$ CFU followed by unidentified fungi with a total of $8.5x10^4$, Streptococci with a total of $4.3x10^4$ CFU and *S. aureus* with a total of $1.2x10^4$ from all surgical gears sampled. Gram +ve bacilli and *P. acnes* were isolated in smaller numbers with a total, $3.5x10^3$ Gram positive bacilli and $6.3x10^3$ *P. acnes*.

Table 4-3 Isolated organisms identified from each HP part

Handpiece part	Identified organisms	Number Isolated (CFU/instrument)	Total Handpiece Isolates (CFU/instrument)
High speed turbine	*Leuconostoc* spp.	$1x10^2$	$4.7x10^4$
	Gamella morbillorum	$1x10^2$	
	Streptococcus mutans	$1x10^2$	
	Streptococcus gordonii	$1x10^2$	
	Pseudomonas stutzeri	$7x10^3$	
	Pseudomonas melocina	$1x10^2$	
Low speed spray channel	*Streptococcus salivarius*	$4.4x10^3$	$3.9x10^4$
	Streptococcus oralis	$2x10^2$	
	Streptococcus mitis	$2x10^2$	
	Staphylococcus aureus	$4.6x10^3$	
Surgical gear	*Streptococcus salivarius*	$5.3x10^2$	$2.4x10^5$
	Leuconostoc spp.	$5x10^2$	
	Streptococcous sanguinis	$5.1x10^3$	
	Streptococcus gordonii	$1.8x10^2$	
	Streptococcus mitis	$2.2x10^4$	
	Streptococcus oralis	$1.4x10^4$	
	Streptococcus mutans	$1x10^4$	

Table 4-4 Number of *P.acnes* types isolated from GDH dental HP's

***P.acnes* types**	**Number of *P.acnes* types isolates.**
IA	1
IB	10
II	0

4.2.5 *Microbial contamination of repair facility dental handpieces*

A total of 24 W&H TA-98 high speed HP's, 14 W&H TA-97, 4 W&H TopAir high speed HP's, 2 W&H WA56 low speed HP's and 2 W&H S11 surgical HP's were sampled. These HP's were sent for maintenance and repair and each included a certificate indicating the HP had been through a decontamination process though these decontamination processes were unknown.

No bacteria were isolated from the negative controls. Bacteria were isolated from a total of 20/24 TA- 98 turbines (median 40 CFU/instrument, range 0 – $2.9x10^3$ CFU/instrument), 12/14 TA -97 turbines (median 140 CFU/instrument, range 0 – $1.9x10^3$ CFU/instrument), 4 Topair turbines (median 50 CFU/instrument, range 20 – 140 CFU/instrument), 2 S11 gears (median $1.7x10^3$ CFU/ instrument, range 150 – $3.4x10^3$ CFU/instrument) and 2 WA 56 spray channels (median 270 CFU/instrument, range 40 – 500 CFU/instrument) (Figure 4-8). The majority of organisms isolated from each HP location were CONS whilst Gram positive bacilli including *Bacillus spp, S. aureus*, *P. acnes* and oral *Streptococci* were also identified (Figure 4-8, Figure 4-9, Figure 4-10, Figure 4-11, Table 4-5). A total of 12 *P. acnes* isolates from repair facility HP's were identified by typing (Table 4-6).

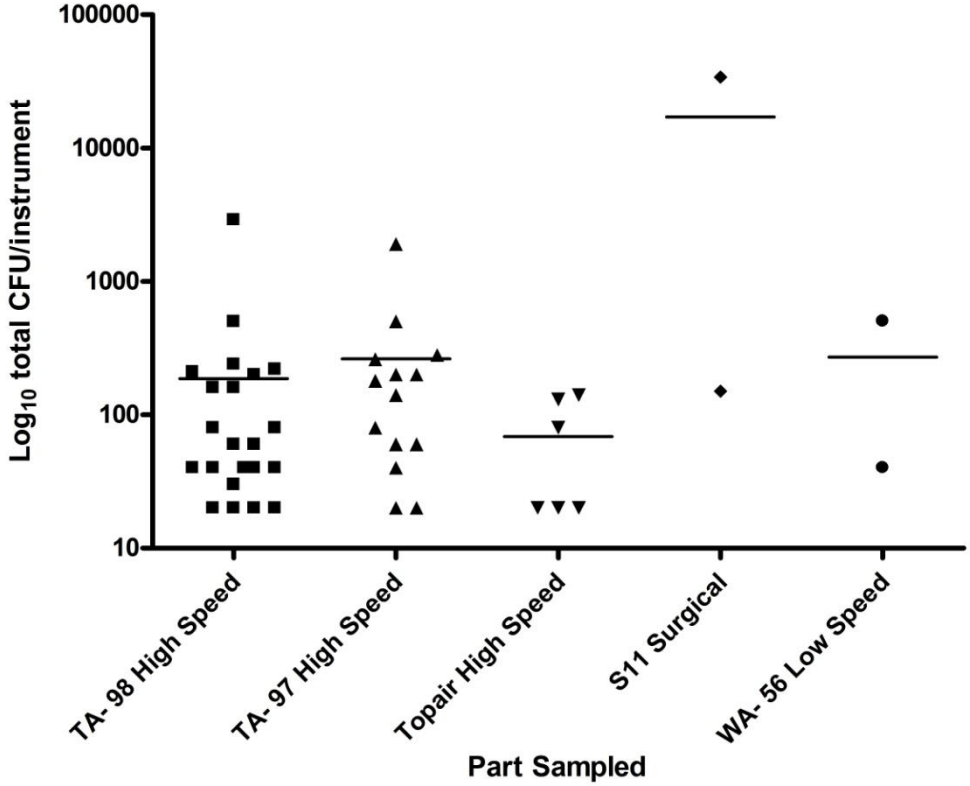


Figure 4-7 Number of bacteria isolated from each repair facility HP part. Internal HP parts were sampled for aerobic and facultative anaerobic isolates. The turbine of high speed HPs, the spray channel of the low speed HP and the gear of surgical HPs were sampled. Each part was cultured for aerobic and facultative anaerobic isolates. A single surgical gear had the highest numbers of bacteria with a value of $3x10^4$ CFU. The mean of datasets is represented by a horizontal line.

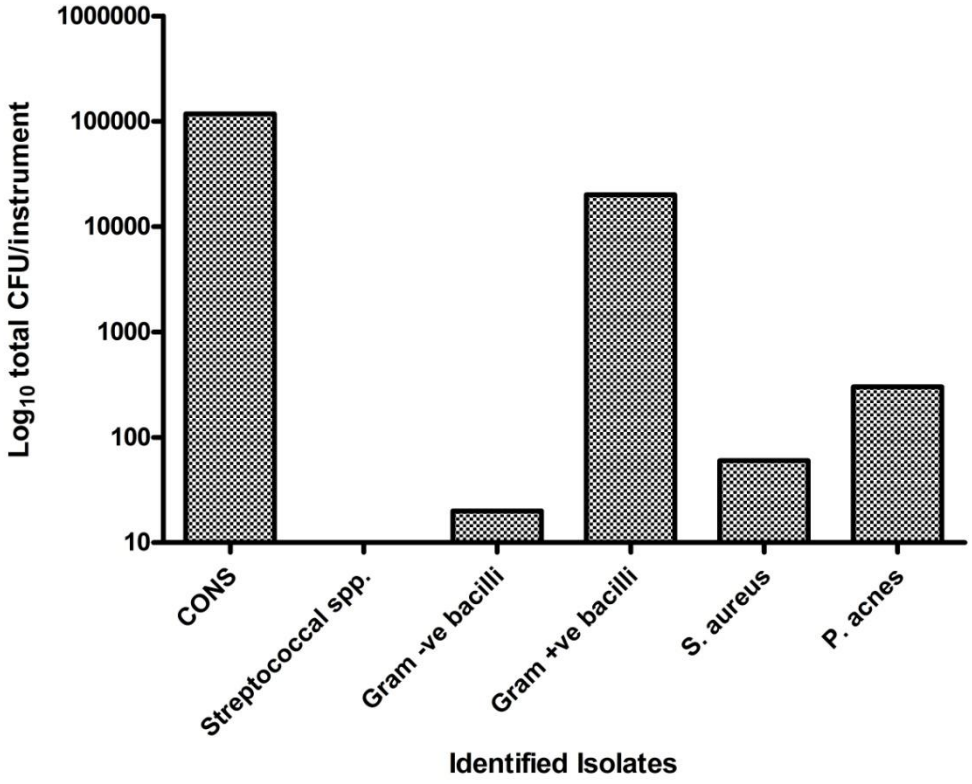


Figure 4-8 Number of Isolates from TA-98 HP's from repair facility. Bacteria isolated from the high speed turbines were identified using diagnostic microbiology techniques. The majority of bacteria isolated were CONS with a total of $8.5x10^5$ CFU isolated and Gram positive bacilli with a total of $2x10^4$ CFU from all turbines sampled. Gram negative bacilli and *P. acnes* were isolated in smaller numbers with a total of 20 CFU for Gram -ve bacilli and $1x10^3$ *P. acnes* isolated.

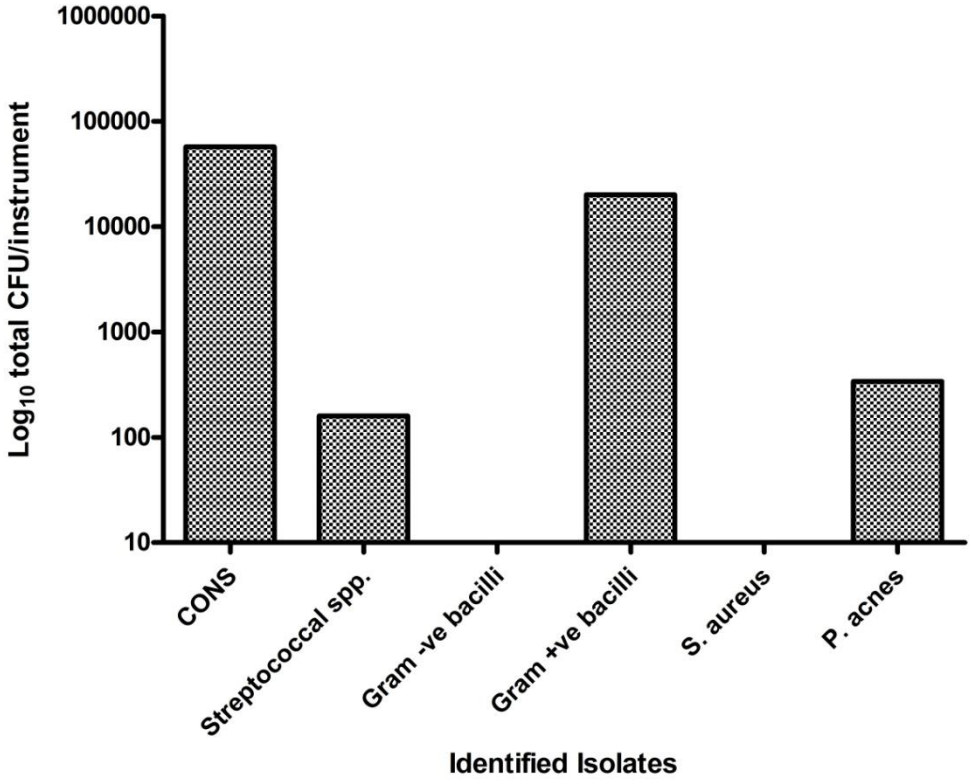


Figure 4-9 Number of Isolates from TA-97 HP's from repair facility. Bacteria isolated from the high speed turbines were identified using diagnostic microbiology techniques. The majority of bacteria isolated were coagulase negative staphylococci (CONS) with a total of $5.6x10^4$ CFU and Gram positive bacilli with a total of $2x10^4$ CFU from all turbines sampled. Streptococcal spp. and *P. acnes* were isolated in smaller numbers with a total of $1.2x10^2$ CFU for Streptococcal spp. and $1.6x10^2$ *P. acnes* isolated.

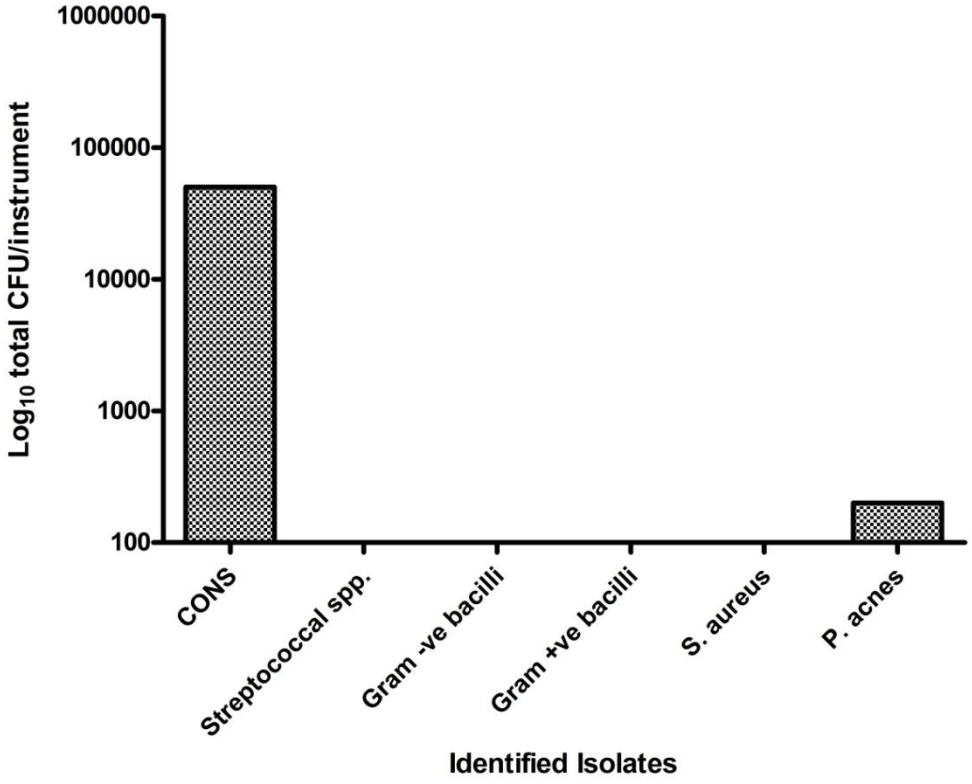


Figure 4-10 Number of isolates from Topair HP's from repair facility. Bacteria isolated from the high speed turbines were identified using diagnostic microbiology techniques. The majority of bacteria isolated were coagulase negative staphylococci (CONS) with a total of $4.9x10^{4.}$ *P. acnes* were isolated in smaller numbers with a total of $2x10^{2}$ CFU.

Table 4-5 Isolated organisms identified from each HP part

Handpiece type	Bacteria identified	Number Isolated (CFU)	Total Handpiece Isolates (CFU)
S11 – Surgical	Coagulase –ve Staphylococci	$3.4x10^4$	$3.4x10^4$
WA 56 Low speed	*S. sanguinis*	20	$5x10^2$
High speed TA 98	*Bacillus firmus,* *Bacillus subtilis,* *S. aureus* *P. acnes*	40 20 $1.2x10^2$ $2x10^2$	$5.2x10^3$
High speed TA 97	*Bacillus pumis* *Gamella morbellorium* *Streptococcus anginosis* *Lactococcus lactis*	$2x10^3$ $1x10^2$ $1x10^2$ $9.8x10^2$	$2.7x10^3$

Table 4-6 Number of *P.acnes* types isolated from repair facility dental HP's

***P.acnes* types**	**Number of *P.acnes* isolates**
IA	3
IB	8
II	1

4.2.6 *16S PCR of handpiece eluents*

Samples that had been previously sampled by culture were sampled using 16S PCR (Section 2.2.6). DNA extracted from a swab of a feline oral cavity was used as a positive control. For decontaminated samples, a used, unprocessed HP sample was used as a positive control. Negative controls comprised ROH_2O and HP negative control eluents for all experiments (Section 4.2.4). No DNA was detected in all negative controls (Table 4-7, Table 4-8, Table 4-9, Table 4-10). For used, unprocessed samples, culture positive surgical HP eluents, all samples contained 16S DNA (Figure 4-11, Table 4-7). For decontaminated turbines, all culture negative samples contained 16S DNA Table 4-8, Figure 4-11). For decontaminated spray channels, both culture positive and culture negative samples all contained 16S DNA (Table 4-9). For decontaminated surgical gears, culture negative samples all contained 16S DNA (Table 4-10).

Table 4-7 Number of bacteria isolated from used,unprocessed samples using culture techniques and DNA concentration extracted from samples.

HP serial number	Blood agar CFU/instrument	FAA agar CFU/instrument	Concentration DNA isolated (ng /ml)	16S DNA (+/-)
Negative Control (DNAse free H_2O)	0	0	0	-
Positive Control (Oral swab)	Not Sampled	Not Sampled	224	+
06508	175	$2.2x10^3$	31.57	+
06374	$1.4x10^3$	875	53.78	+
18007	$1x10^3$	$3 x10^3$	82.3	+
18008	525	175	81	+
06372	$1.9x10^3$	$7.5x10^3$	107	+

Table 4-8 Number of bacteria isolated from decontaminated turbine samples using culture techniques and DNA concentration extracted from samples.

HP Serial number	Blood agar CFU/instrument	FAA agar CFU/instrument	DNA isolated (ng/ml)	16S DNA (+/-)
Positive Control	$1x10^3$	$3 x10^3$	82.3	+
Negative Control (DNAse free H_2O)	Not Sampled	Not Sampled	0	-
Turbine Control	0	0	0.03	-
24643	0	0	2	+
07862	0	0	1.9	+
56960	0	0	2.8	+
19340	0	0	2.1	+
41517	0	0	2.3	+
56867	0	0	1.8	+
09234	0	0	4.1	+
54995	0	0	1.7	+
09639	0	0	1	+
57013	0	0	1.1	+

Table 4-9 Number of bacteria isolated from decontaminated spray channel samples using culture techniques and DNA concentration extracted from samples.

HP Serial number	Blood agar CFU/instrument	FAA agar CFU/instrument	DNA isolated (ng/ml)	16S DNA (+/-)
HP Positive Control	$1x10^3$	$3 x10^3$	82.3	+
Negative Control (ROH_2O)	Not Sampled	Not Sampled	0	-
Spray Channel Control	0	0	0.2	-
53708	0	0	2	+
12385	0	0	1.9	+
05564	420	0	2.8	+
24613	0	0	2.1	+
57825	0	0	2.3	+
19359	240	0	1.8	+
21555	1560	600	4.1	+
03850	120	0	1.7	+
57828	60	0	1	+
58333	0	0	1.3	+

Table 4-10 Number of bacteria isolated from decontaminated surgical gear samples using culture techniques and DNA concentration extracted from samples.

Serial number	Blood agar CFU/instrument	FAA agar CFU/instrument	DNA isolated (ng/ml)	16S DNA (+/-)
Positive Control	$1x10^3$	$3 x10^3$	82.3	+
Negative Control (ROH_2O)	Not Sampled	Not Sampled	0	-
Surgical control	0	0	0.03	-
06365	0	0	2.1	+
18010	0	0	0.7	+
18008	0	0	1.8	+
06390	0	0	2.6	+
06500	0	0	1	+

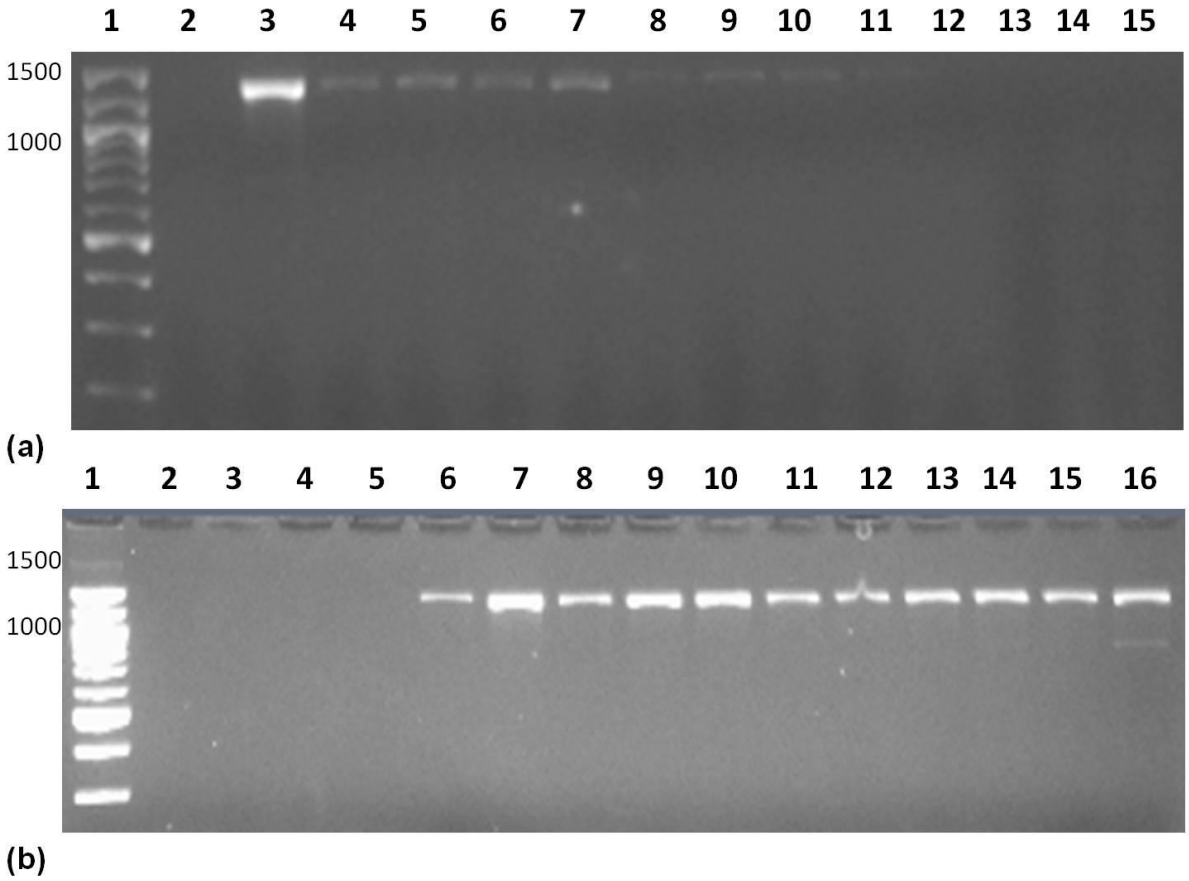


Figure 4-11 16S PCR of used, unprocessed HP's. Eluents from surgical HP's before **(a)** and after the GDH decontamination process **(b)** (Table 2-3 **Details of GDH CSSD decontamination process.** were sampled for the presence of 16S DNA. A single band representing the presence of 16S DNA is observed in the positive control (lane 3) and each HP sample dilution (06372 lanes 4,5,6) (06374 lanes 7,8,9) (06508 lanes 10,11, 12). No bands are visible in the negative control (lane 2) or the HP negative control (lanes 13, 14, 15). Band sizes are indicated in bp by the 100bp ladder (lane 1).

4.3 Discussion

Dental HP's can be used to undertake invasive procedures and although it is the bur of the HP that is in direct contact with pulpal, gingival and alveolar bone; the contaminants are internalised into the HP structure (Lewis et al., 1992). If decontamination procedures are not carried out for internal areas, these contaminants may be released during subsequent reuse in the form of an aerosol that may come into contact with patients or healthcare professionals (Rautemaa et al., 2006). It is for these reasons that the Centres for Disease Control (CDC) recommend cleaning and heat sterilization of the HP after every patient as "desirable" (CDC - Guidelines for infection control in dental healthcare settings 2003).

To partly justify this recommendation, it is important to determine the common contaminants of HP's during routine use. Bacteria are common contaminants of medical and dental instrumentation and are capable of causing disease if instruments are not sterilized (Chin et al., 2006). The ability to form a protective biofilm matrix is associated with increased survivability (Donlan and Costerton, 2002), resistance to decontamination procedures (Stewart et al., 2001) and is of concern when instrument decontamination is considered. Bacteria have been previously isolated from HP turbines, spray channels before and after decontamination processes (Kellett and Holbrook, 1980) and gears of low speed handpieces during routine use (Herd et al., 2007).

To access internal HP parts, dismantling is required which may be difficult without specialist training and tools. Some parts are also challenging to access without damage to the HP and ensure no contamination during sampling. Previous studies have therefore relied on the application of swabbing of easily accessible gears (Herd et al., 2007) or flushing sterile solution through an intact HP to remove internal bacteria (Kellett and Holbrook, 1980). The sensitivity of both methods was not stated though the swabbing technique has a variation of reported sensitivities from 19% (Angelotti et al., 1964) to 87% (Buttner et al., 2001) for spore recovery.

This study utilised a sonication method which has been previously used for desorption of bacteria from hip joints (Tunney et al., 1998), catheters (Gorman et al., 1994), and stents (Keane et al., 1994). Validation work undertaken in this study has shown that sonication represents an increase in sensitivity when compared to swabbing methods used in previous studies (Bjerkan et al., 2009, Herd et al., 2007). This is also reflected in the increased number and species of bacteria isolated from each location when compared to previous studies with Chin et al. (2006) isolating a median of 547 CFU and Kellet et al. (1980) isolating a median of $1.1x10^3$ cfu /HP from the spray channel and the turbine by rinsing. Whilst the sonication method can only sample the entire part rather than targeting specific areas of the surface, the sonication method represents an improvement in sensitivity and therefore more realistic numbers and species of bacterial contamination of dental HP parts. The removal of bacteria from the narrow lumened spray channel by sonication may not be as efficient as the removal from a flat, open surface. Previous studies have utilised flushing of the lumen with a sterile saline solution (Kellett and Holbrook, 1980)) and the insertion of floss directly into the channels (Martiny and Simonis, 2009). The sensitivity and reproducibility of these methods was not validated in the studies and sampling of lumens may still present a difficulty.

Dental HP's from the GDH were sampled before and after decontamination. Validation experiments indicated that lubricating oil, present before and after decontamination, did not affect the growth of a range of common HP contaminants. Cleaning solution, used before the sterilization process, affects the viability of microorganisms as would be expected due to the presence of alcohol in the solution.

Before decontamination, HP's were acquired after transport to the CSSD which was not undertaken in aseptic conditions until transport to the laboratory. This study was concerned with the challenge to the HP before decontamination including any environmental bacteria. Sampling of the negative control HP's indicated that no bacteria isolated could be attributed to the passage through the CSSD facility or by experimental processing. In the repair facility, aseptic techniques were used due to the lack of a laminar flow cabinet. Negative controls performed during sampling also indicate that processing using this method resulted in no contamination from the environment.

All HP's sampled were selected to be the latest models available at the GDH to partly standardise the age of the HP's. Modern HP's also have design features such as the hygienic head system to prevent contamination entering the HP head. Despite the presence of these systems, bacteria were still isolated from each HP location sampled. Before decontamination, the majority of bacteria isolated from each GDH HP were CONS which are associated with the oral cavity and the environment (Jackson et al., 1999). These may cause opportunistic infections and have previously been isolated from endodontic lesions (Niazi et al., 2010). Viridians *Streptococci* including *S. mutans, S. oralis* and *S. sanguinis* are associated with the oral cavity and dental caries (Hintao et al., 2007). The identified *Pseudomonas spp., P. stutzeri* and *P. mendocina* have been previously isolated from contaminated dental unit waterlines (Singh et al., 2003) and associated with opportunistic infections (Noble and Overman, 1994, Zaluski et al., 1999).

P. acnes has been associated from the oral cavity and human skin (McDowell et al., 2005) where it can cause infections such as acne (Burkhart and Burkhart, 2006) or inflammatory disease in the body if it is present in the bloodstream (Bayston et al., 2007a). *P. acnes* have also been associated with prosthetic hip joint infections and endocarditis (Tunney et al., 1998, Vanagt et al., 2004). *P. acnes* has also been isolated from endodontic lesions along with CONS (Niazi et al., 2010), which has implications for the spread of dental infections. The majority of *P.acnes* isolates from the repair facility and the GDH HP's were type I which is consistent with types previously isolated from dental infections such as periodontitis (McDowell et al., 2005). The first differences between *P. acnes* types were first characterised by differences in agglutination tests and differences in cell wall sugars (Johnson and Cummins, 1972). Molecular typing of the *rec*A gene in isolates has further differentiated the isolates further into type IA and type IB.It is hypothesised that different *P. acnes* types produce different virulence factors with type IB strains associated with a greater expression of co haemolytic cyclic adenosine monophosphate factor associated with haemolytic activity (Valanne et al., 2005). A further study into the protein expression by *P. acnes* types revealed only 2 proteins that are distinct for type I isolates, a conserved hypothetical protein with homology to a *Corynebacterium* protein, an unidentified protease, and a hypothetical protein specific to *P. acnes* (Holland et

al., 2010). Whilst it is not clear if expression of these proteins results in greater virulence, all *P. acnes* isolates produce proteins associated with tissue destruction and inflammation (Holland et al., 2010). The surgical HP gear had the most abundant number of microorganisms of the parts sampled which might be due to the larger surface area of the gear and the fact that the surgical HP is used to undertake more invasive procedures and therefore be exposed to more microorganisms. It is interesting to speculate that the source of nosocomial *P.acnes* and CONS causing endodontic infections could be incomplete decontamination of dental HP's.

After decontamination, Gram-negative bacilli were the predominant bacteria isolated from low speed spray channels, and *P. acnes* were also isolated along with a smaller number of CONS. This flora selected out by the decontamination process might be due to the change in environment brought about by decontamination, may have been flora particular to the HP's sampled or may have come from the HP lubricating oil if microorganisms are able to survive upon exposure. Lubricating oil may not allow steam penetration of the HP and has been shown to inhibit steam sterilization (Lewis and Boe, 1992). Whilst the spray channel is not often required for routine low speed HP use and the number of bacteria isolated were significantly reduced; the presence of bacteria indicate the lack of a sterile instrument. The bacteria isolated are capable of causing opportunistic disease and may contribute to cross infection between patients and represent a marker of a failure in the decontamination process. This finding requires further investigation including sampling of the environment or the lubricating oil and a larger number of HP's to determine if this is a false positive or an indicator of a deficiency in the decontamination process.

The repair facility HP's had significantly more CONS than the GDH HP's. HP's sent for repair were sourced from both hospitals and dental practice and it was not clear how HP's from each location correlated with the results due to the need for anonymity. Whilst each HP sent for repair came with documentation stating that each device has been through a decontamination process, this could not be verified and decontamination process may vary depending on the HP source (Smith et al., 2009) Further studies into HP's used and decontaminated in dental practices may present different results to that found with GDH HP's that are decontaminated by the current best practices for instrument

decontamination (Smith et al., 2009). The bacterial species isolated whilst being similar to the GDH isolates, also contained more Bacillus spp., less *Streptococcal* spp. and *L. lactus* that is associated with dental caries (Marchant et al., 2001). The number of bacteria isolated clearly indicates a failure of decontamination processes. All HP's were not in working condition and any blockages may inhibit routine cleaning processes or steam sterilization. The findings highlight the need for routine HP maintenance to prevent the proliferation of bacteria.

The findings presented herein are similar to the only previous study to identify HP microbial contamination with CONS, oral *Streptococci, Pseudomonas spp* and *Bacillus spp* among the microorganisms isolated (Kellett and Holbrook, 1980). The CONS isolates in this study were not identified beyond the species level and consisted of any Gram positive cocci that were also catalase positive and coagulase negative. Therefore CONS isolates represented a number of different species though all with similar resistance to the decontamination process and characteristics of survival in the environment. Fungi were also only identified to species level before decontamination. The detection of no bacteria does not mean that an HP is free of microorganisms and therefore sterile. The survey for microorganisms may have been limited by the isolation methods used for culture of organisms. The BA plates were found to grow mostly CONS, and FAA incubated anaerobically that oral *Streptococci* and other organisms such as *P. acnes* were observed. The use of other media may have allowed for the growth of other microorganisms that are outcompeted by the fast growing *Staphylococci*. The oral cavity is also home to fastidious or non culturable bacteria which would be missed when culture techniques alone are employed (Hamlet, 2010). It is for this reason that 16S PCR was employed that detects DNA sequences specific for bacteria. For the GDH isolates, 16S PCR confirmed the presence of bacterial DNA in culture positive samples for validation of the method which was also be applied to culture negative samples. All decontaminated HP samples, including culture negative samples, were positive for 16S DNA. The presence of 16S DNA does not indicate viable bacteria and the use of real time PCR for specific organisms will allow the measurement of gene expression and therefore the viability of any targeted organisms (Hamlet, 2010).

The study did not attempt to identify viral DNA. The PCR techniques have also been utilised previously to identify viral DNA from dental HP's after operation on

patients infected with HIV (Lewis and Boe, 1992) and hepatitis B (Hu et al., 2007b). Again, the detection of viral DNA does not indicate the presence of infectious viruses and the detection of viral DNA could be combined with cell culture studies to measure infectivity of cells and therefore indicate the survival of viruses (Epstein et al., 1995).

The results before HP reprocessing can inform development of HP decontamination processes and can result in test soils comparable to *in vivo* biofouling for HP reprocessing technologies. Of the current test soils accepted by the European standard BS ISO/TS, only the German and American test soil contains a bacterial challenge. The German test soil utilizes a culture of 10^{11} CFU/ml suspension of *Enterococcus faecium* and the American test soil contains *Bacillus atrophaeus* spores. A test soil specific for dental HP's could be designed with knowledge of the bacterial species and counts detailed in this study and provide a test challenge closer to that found *in vivo* for any new cleaning technologies.

In conclusion, dental HP's are contaminated with environmental and oral bacteria before and after decontamination in hospital and repair facilities. The presence of bacteria in HP's after decontamination procedures is a concern and routine decontamination procedures of dental HP's may not be adequate if the aim is to deliver a sterile instrument at the point of use. Further studies utilising culture independent techniques would allow a more complete picture of HP microbial contamination and further detail the cross infection risk to patients and healthcare professionals.

5 Chapter 5: Quantitative and Qualitative Analysis of Protein Contamination of Dental Instruments

5.1 Introduction

The decontamination processes of invasive medical instruments are under constant review as new challenges to instrument reprocessing emerge (Sehulster, 2004). Research into decontamination processes has recently incorporated research into protein contamination of instruments (Lipscomb et al., 2006b), rather than focusing on the presence or absence of bacteria alone (Lewis et al., 1992). Protein contamination can increase the dissolution of metal ions by complexing metal ions with proteins which causes corrosion of instrument stainless steel (Williams et al., 1988, Kocijan et al., 2003). In addition, residual protein conditioning film may promote the adhesion of bacteria through specific adhesion receptors, such as fibronectin binding protein found in *Staphylococcus aureus* (Piroth et al., 2008). Protein can also inhibit the sterilization process if not removed during instrument cleaning (Parker and Johnson, 1995).

The emergence of the heat and chemical resistant prion protein, the causative agent of CJD, has highlighted the importance of research into protein contamination of medical instruments. Protein contamination must be removed by the cleaning stage of the instrument reprocessing cycle (Lipscomb et al., 2007), as the subsequent disinfection and sterilization stages may fix protein contamination onto the surface (Nakata et al., 2007). It is for this reason that protein contamination is used as a marker for cleanliness of instruments and for the validation of cleaning procedures and technologies. The European standard BS-EN-ISO-15883 part 1 details protein assays for the detection of protein and a failure of cleaning is indicated by a positive reaction with one of the assays. Concerns have recently been raised as to the sensitivity of these protein tests and how sensitive a test for protein cleanliness should be (Lipscomb et al., 2006a).

A method of answering this question would be to undertake a qualitative analysis of typical instrument protein contamination. Whilst quantitative data provides some insight into instrument contamination levels, a qualitative analysis would put this information into context by identifying common contaminants that must be removed by the cleaning process and inform of any cross contamination risk

derived from protein contamination. There is currently a lack of published literature on qualitative analysis of instrument protein contamination.

A qualitative analysis is possible by desorption of protein from the instrument followed by precipitation of the sample to allow viewing of the proteins by SDS PAGE. The SDS–PAGE method allows the separation of protein by size to allow identification by MS or Western blotting.

This study aimed to undertake validation work to develop a method that would allow the extraction, precipitation and detection of protein from instrument surfaces before employing these techniques to undertake a quantitative and qualitative analysis of protein contamination of dental extraction forceps and dental HP’s.

5.2 **Results**

5.2.1 ***Validation of protein extraction methods***

To assess the sensitivity of 1% (v/v) SDS solution, 1% (v/v) Decon® 90 (Decon® Laboratories, Hove, UK) and a 0.1M NaOH solution (Fisher scientific, Loughborough UK) at removing BSA from a SSS, a total of 9 prepared SSS's (Section 2.5.1.1) were inoculated with a 10 µl solution of 100 µg of BSA. These were placed in a clear flat bottomed Costar™ 24 well plate and air dried for 16 h at room temperature of 22°C representing the length of time instruments can be left for central reprocessing (Plinston et al., 2007). Prepared SSS's were incolulated with 10 µl of ROH_2O as controls. All SSS's were inserted into sterile Bijoux tubes (Sterilin, Caeriphilly UK). SSS's inoculated with protein and a negative control were immersed in 1 ml of 1% (v/v) SDS solution, 1 ml of 1% (v/v) Decon™ 90 or 0.1M NaOH 0.1 pH 9.2. The remaining SSS's and negative control were immersed in Decon™ 90 solution (Smith et al., 2005). Bijoux tubes containing 1% (v/v) Decon™ 90 and 1% (v/v) SDS were inserted into a Fisherbrand® 11021 sonic bath (Fisher Scientific, Loughborough UK) filled with ROH_2O and sonicated for 30 min at 35 kHz (Smith et al., 2005). Tubes containing 0.1M NaOH and 1% (v/v) SDS was immersed in a water bath (Grant Instruments, Cambridge UK) containing ROH_2O and boiled for 10 min at 100°C (Imamura et al., 2003). Each eluent was sampled for protein using the OPA assay (Section 2.3.1.3) and SDS- PAGE (Section 2.3.3).

No protein was isolated from the negative controls (n=3). Boiling in 1% (v/v) SDS resulted in more protein recovery than 1% (v/v) Decon, 0.1M NaOH and sonication in 1% (v/v) SDS (

Table 5-1). Boiling in 0.1M NaOH resulted in the least protein recovery of all methods sampled. BSA recovery from the stainless steel surface by boiling and sonication in 1% (v/v) SDS and sonication in 1% Decon was visualised by SDS-PAGE with BSA being visible in all samples (Figure 5-1).

Table 5-1 Efficacy of protein extraction techniques and detergents.

Detergent	Extraction Process	Median protein recovery (µg/ml)	Range of protein recovery (µg/ml)
1 % (v/v) SDS	Sonication	80	63.7 – 94.9
	Boiling	87	82 – 94
1 % (v/v) Decon®90	Sonication	57	22.8 – 77.8
0.1M NaOH	Boiling	53	50 – 66.5

The data shown is the median of three experiments.

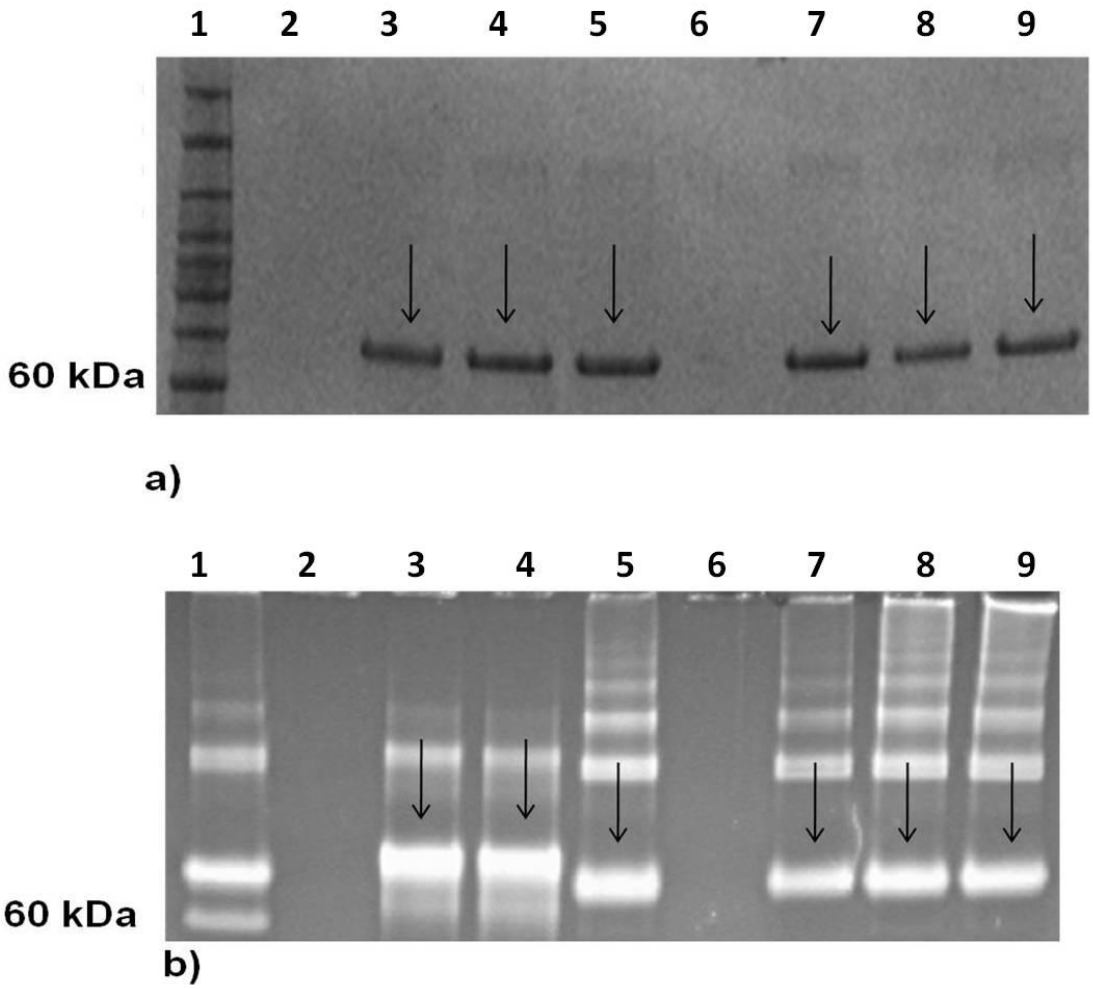


Figure 5-1 Recovery of BSA from SSS's using different cleaning solutions and methods. Samples from SSS's inoculated with BSA and extracted using each method were separated using 4-12% Bis-Tris gels. Gel **(a)** was stained with Coomassie blue and gel **(b)** was stained using SYPRO® Ruby. BSA inoculated and dried on SSS's was recovered by boiling and sonication in 1% (v/v) SDS **(a)** Both boiling (lanes 3, 4, 5) and sonication (lanes 7, 8, 9) in 1% (v/v) SDS recover BSA fractions indicated by arrows from SSS's. No protein was visible in the negative control SSS eluents (lane 2 and lane 6). Lane 1 contains the protein size ladder for comparison with the closest size to the fraction V BSA protein indicated in kDa. BSA was also recovered by sonication in 1% (v/v) Decon®90 and 1% (v/v) SDS **(b)** Both 1% (v/v) Decon®90 (lanes 3,4,5) and 1% (v/v) SDS (lanes 7, 8, 9) recover BSA from the surface as shown by comparison to the positive control (lane 1). No protein is visible in the negative control disc eluents (lanes 2 and 6).

5.2.2 *Validation of protein precipitation methods*

To assess the protein recovery of each precipitation method (Section 2.3.2.1, Section 2.3.2.2, Section 2.3.2.3, Section 2.3.2.4), a total of 3 x 2 ml 50 µg/ml solutions of BSA in 1% (v/v) SDS were sampled for each precipitation method representing the sample volume that HP turbines are immersed (Section 2.2.4). A 2ml solution of 1% (v/v) SDS was also prepared as a negative control. The solutions were each precipitated and protein sampled using the OPA assay (Section 2.3.1.3) and SDS PAGE (Section 2.3.3).

The Amicon® Filter method resulted in the recovery of the most median protein of all the methods sampled and the TCA method recovered the least median protein (Table 5-2). The precipitation of BSA by acetone, TCA, StrataClean™ resin and Amicon® filtration were visualised using SDS–PAGE (Figure 5-2).

Table 5-2 Recovery of protein by each precipitation method.

Precipitation method	Median protein recovered (µg/ml)	Range protein recovered (µg/ml)
Acetone	15.1	8.6 – 24
TCA	13	10 – 15
StrataClean® Resin	23.9	19.5 – 24.2
Amicon® Filter	30.1	29.8- 31.8

Data shown is the median of 3 experiments.

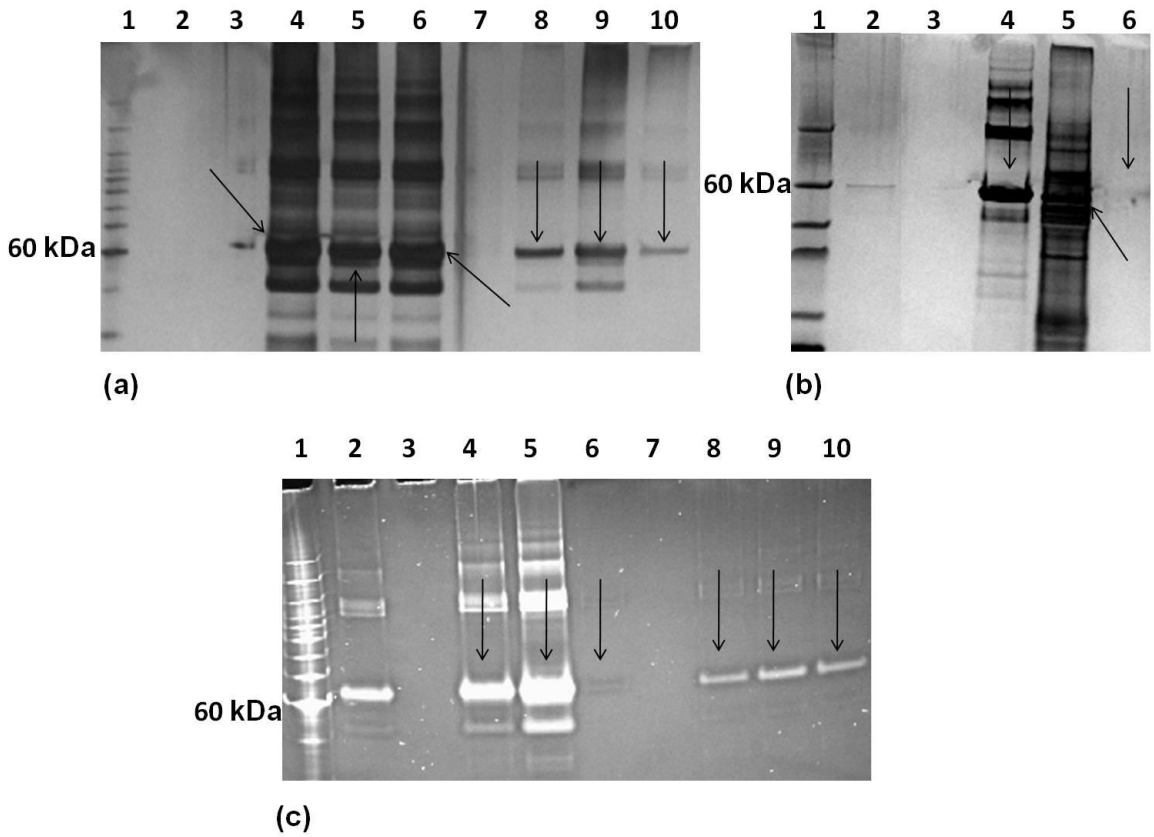


Figure 5-2 Precipitation of BSA by different methods. Samples of precipitated 50 µg/ml BSA solutions were separated using 4-12% Bis-Tris gels. Gel **(a)** and gel **(b)** were stained using silver stain and gel **(c)** was stained using SYPRO® Ruby. BSA was precipitated using TCA and acetone **(a)** BSA fraction V indicated by arrows was recovered by acetone precipitation (lanes 4, 5, 6) and TCA precipitation (lanes 8, 9. 10). No protein was observed in the negative controls (Lanes 2, 3, 7). Lane 1 contains the protein ladder with sizes indicated in kDa for comparison. BSA was also precipitated using Amicon® filtration **(b)** precipitated the BSA solutions at varying concentrations (lanes 4,5,6). No protein was observed in the negative controls (lane 3) An unprecipitated 50 µg/ ml BSA positive control was included for comparison (lane 2) and lane 1 contains the protein ladder with sizes indicated in kDa for size comparison. Both acetone and StrataClean™ Resin precipitated BSA **(c)**. Acetone (lanes 4, 5, 6) and StrataClean™ Resin (lanes 8, 9, 10) precipitated BSA though at varying concentrations. No protein was observed in the negative controls (lanes 3 and 7). A 50 µg BSA positive control was included for comparison (lane 2) and lane 1 contains the protein ladder with sizes indicated in kDa for size comparison.

5.2.3 *Sensitivity of SDS-PAGE staining*

To determine the sensitivity of each protein staining technique (Section 2.3.4.1, Section 2.3.4.3, Section 2.3.4.4),BSA was diluted serial ten fold from 10^{-5} to 100 µg/ml in 1% (v/v) SDS and a 20 µl sample of each concentration was processed for SDS PAGE (Section 2.3.3). To determine the sensitivity of PAS staining, salivary mucin was also diluted serial ten fold from 10^{-5} to 100 µg/ml. Each gel was stained with Coomassie blue staining (Section 2.3.4.1), silver stain (Section 2.3.4.3) and PAS staining (Section 2.3.4.4) to determine the sensitivities of both staining methods. Sensitivity was determined using Quantity One® software (Bio-Rad version 4.6.7) to determine the presence of contrast peaks indicating the presence of protein bands.

Coomassie blue staining sensitivity for detecting salivary amylase was calculated as 0.2 µg of protein (Figure 5-3 [a]). Silver staining had a sensitivity of 0.02 ng of BSA (Figure 5-3 [b]). Periodic acid Schiff reagent stained a total of 0.2 µg of salivary mucin protein (Figure 5-3 [c]).

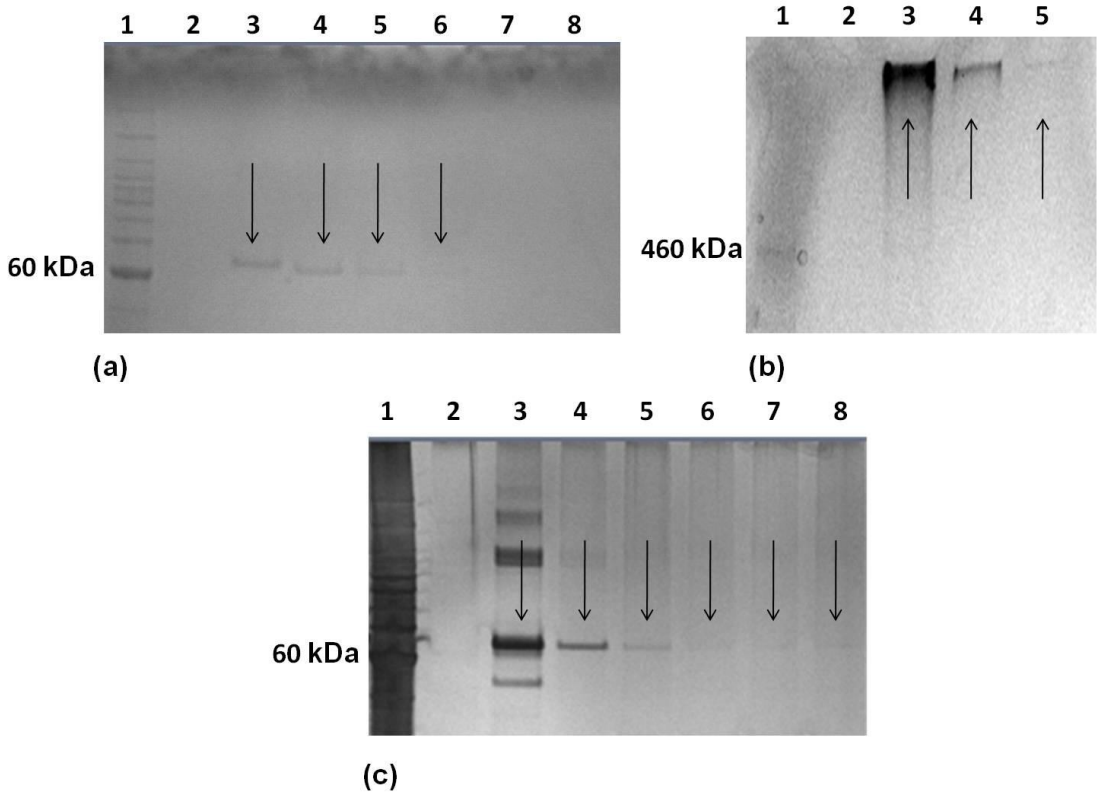


Figure 5-3 Sensitivity of protein detection stains at detecting BSA. BSA of decreasing concentrations were loaded into a 4-12 % Bis Tris gel and stained with coomassie brilliant blue and sliver stain whilst decreasing concentrations of salivary mucin samples were loaded into a 3-8% Tris Acetate gel was used to determine the sensitivity of PAS staining. For coomassie blue staining **(a)**, protein was observed in the 100 µg/ml sample (lane 4), 10 µg/ml sample (lane 5), 1 µg/ml sample (lane 6). No protein was detected in the 0.1 µg/ml (lane 7), 0.01 µg/ml (lane 8), 0.001 µg/ml (lane 9), or the 0.0001 µg/ml (lane 10). No protein was detected in the negative control (lane 2). Lane 1 contained the protein ladder for size comparison and lane 3 contained a salivary amylase positive control. For silver staining **(b)** protein is observed in the 100 µg/ml sample (lane 4), 10 µg/ml sample (lane 5), 1 µg/ml sample (lane 6) the 0.1 µg/ml (lane 7), 0.01 µg/ml (lane 8), 0.001 µg/ml (lane 9), and the 0.0001 µg/ml (lane 10). No protein was detected in the negative control (lane 2). Lane 1 contained the protein ladder for size comparison. For PAS staining **(c)**, salivary mucin was observed at concentrations of 1 mg/ml (lane 3), 100 µg/ml (lane 4), 10 µg/ml (lane 5). No protein was detected in the negative control (lane 2). The protein ladder for size comparison was included in lane 1 with sizes indicated in kDa

5.2.4 *Effect of handpiece lubricating oil on SDS- PAGE*

To determine the effect of HP lubricating oil has on SDS-PAGE, a total of 3 BSA solutions were diluted to a concentration of 100 µg/ml in 1% (v/v) SDS alone or in a 1:1 solution of lubricating oil and 1% (v/v) SDS. Each solution was sampled for SDS PAGE (Section 2.3.3) and stained with SYPRO® ruby stain (2.3.4.2).

BSA was detected in the 1:1 lubricating oil 1 % (v/v) SDS solutions and the 1% (v/v) SDS solutions (Figure 5-4).

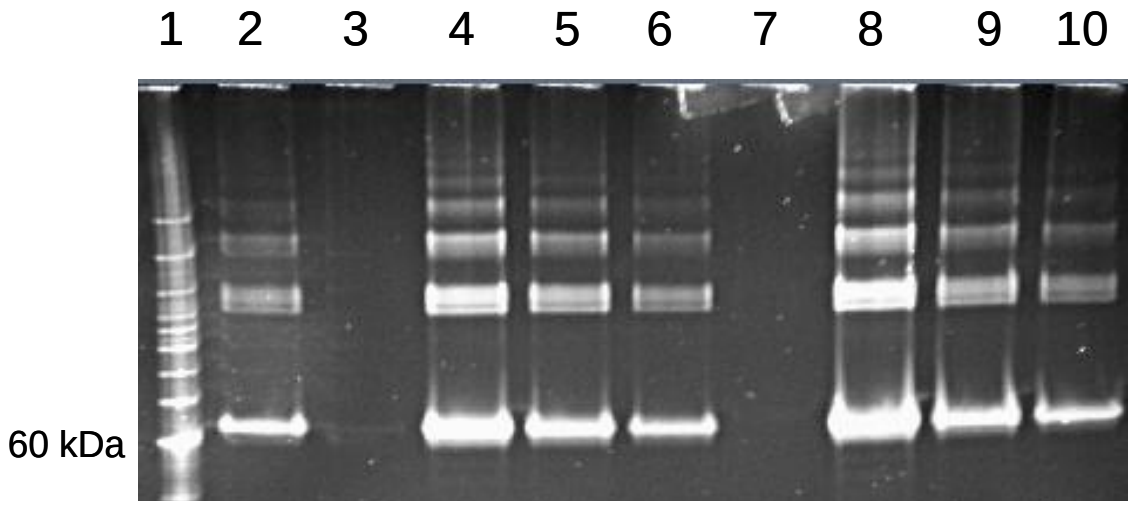


Figure 5-4 Effect of lubricating oil on protein observation by SDS – PAGE. BSA solutions were diluted in 50% solution of lubricating oil and the eluents separated in a 4-12 % Bis Tris gel was stained using SYPRO® Ruby. BSA is visible in solutions of 1% (v/v) SDS (lanes 8-10) and lubricating oil (lanes 4-6) and the positive control of a 100 µg BSA solution in ROH_2O (lane 2). No protein is observed in the negative controls (lanes 3,7). Lane 1 contained the protein ladder for size comparison with sizes indicated in kDa.

5.2.5 *Sensitivity of Antibody Staining and Western Blot*

To determine the sensitivity of antibody probing and determine the most sensitive antibody concentration, salivary amylase was 10 fold serially diluted in 1% (v/v) SDS to concentrations of 0.01, 0.1, 1, 10, and 48 µg/ml and a 10 µl sample of each concentration was added to a polyvinylidene fluoride (PVDF) membrane and dried for 2h at room temperature The strips were also inoculated with a 10 µl sample of 50 µg/ml of BSA as a negative control. Serum albumin was 10 fold serially diluted in 1% (v/v) SDS to concentrations of 0.01, 0.1, 1 and 100 µg/ml and a 20 µl sample of each concentration was added to a PVDF membrane. The strip was also inoculated with 20 µg sample of salivary amylase as a negative control. The strip was probed with antibodies and developed using BCIP/NBT solution (Section 2.3.6). To determine the sensitivity of the Western blot technique, samples of salivary amylase were diluted to concentrations of 10^{-4} to 20 and 40 µg/ml, human serum albumin was 10 fold serially diluted from a concentration of 10-4 to 100 µg/ml and for the sensitivity of the salivary mucin Western blot, unstimulated saliva was processed as in Section 3.2.2 and was diluted 10^{-4}, unprocessed saliva was also diluted 10^{-4} with all dilutions sampled.

A minimum protein amount of 0.002 µg of salivary amylase (Figure 5-5) and 0.0002 µg of serum albumin was detected using antibody probing and alkaline phosphatise staining. The total salivary amylase detected was 0.02 µg (Figure 5-6 [a]) and a total of 0.002 µg (Figure 5-6 [b]) of serum albumin was detected by Western blot. A minimum of a 1/10 dilution of unprocessed saliva and the undiluted processed saliva was also detected (Figure 5-6 [c]).

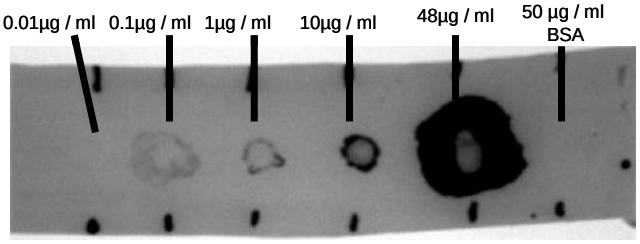


Figure 5-5 Sensitivity of salivary amylase detection using antibody probing. Decreasing concentrations of salivary amylase were added to a PVDF membrane and probed with rabbit anti human IgG followed be secondary probing with goat anti rabbit IgG antibodies conjugated with alkaline phosphatase. The membranes were stained with BCIP/NBT solution. Protein was detected from the 0.1, 1, 10, and 48 µg/ml solutions. No protein was detected on the 0.01 µg/ml solution and the negative control of 50 µg/ml BSA.

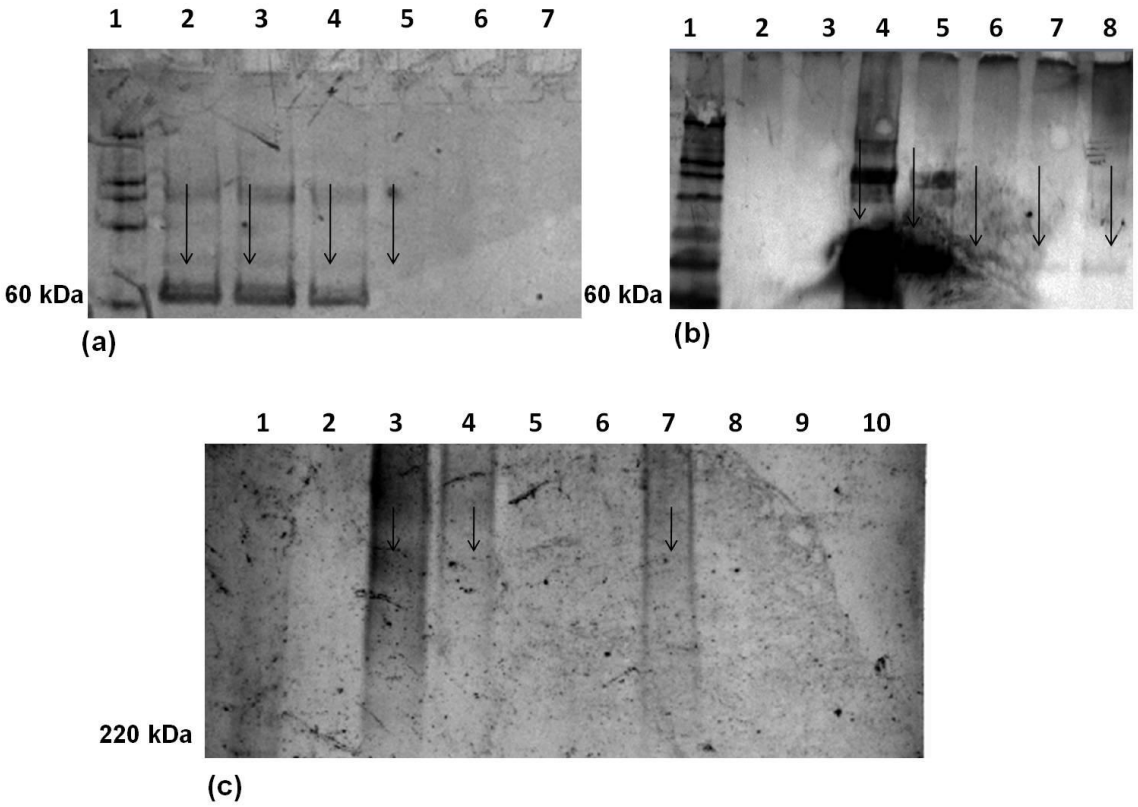


Figure 5-6 Sensitivity of Western blot. Decreasing concentrations of salivary amylase, serum albumin and saliva samples were separated using SDS PAGE, transferred to a PVDF membrane and probed using alkaline phosphatase conjugated antibodies. Salivary amylase **(a)** indicated with arrows was detected in the 40 µg/ml sample (lane 2), the 20 µg/ml sample (lane 3), the 1 µg/ml sample (lane 4) and the 0.1 µg/ml sample (lane 5). No salivary amylase was detected in the 0.01 µg/ml sample (lane 6), 0.001 µg/ml sample (lane 7), 0.0001 µg/ml (lane 8) and the negative control of 100 µg/ml BSA (lane 10). Lane 1 contained the protein ladder for size comparison. Serum albumin **(b)** indicated with arrows was detected in the 100 µg/ml sample (lane 4), the 10 µg/ml sample (lane 5), the 1µg/ml sample (lane 6) the 0.1 µg/ml sample (lane 7), and the 0.01 µg/ml sample (lane 8). No serum albumin was detected in the 0.001 µg /ml sample (lane 9), 0.0001 µg/ml sample (lane 10) and the negative controls of 1% (v/v) SDS (lane 2) and 40 µg/ml of salivary amylase, (lane 3). The salivary mucin **(c)** antibody detected salivary mucin in a neat and a 1/10 dilution of unprocessed saliva (Lanes 3, 4) and a neat solution of processed saliva (Lane 7). No mucin was detected in the 1/100 dilution or 1/1000 dilution (Lanes 5, 6) or the 1/10, 1/100, 1/1000 dilution of the prepared saliva (Lanes 8, 9, 10) or the negative control (lane 2).

5.2.6 *Detection of salivary amylase from saliva samples*

A 5 ml solution of unstimulated saliva was collected from 3 human male volunteers separately. Each sample was centrifuged for 15 min at 9971 *g* and the supernatant removed. Each sample was given a 1:10 dilution and samples sampled using SDS-PAGE (Section 2.3.3). The salivary samples were then probed for the presence of salivary amylase using Western blot (Section 2.3.6).

Salivary amylase was detected from 3 saliva samples and 1 dilute saliva sample (Figure 5-7).

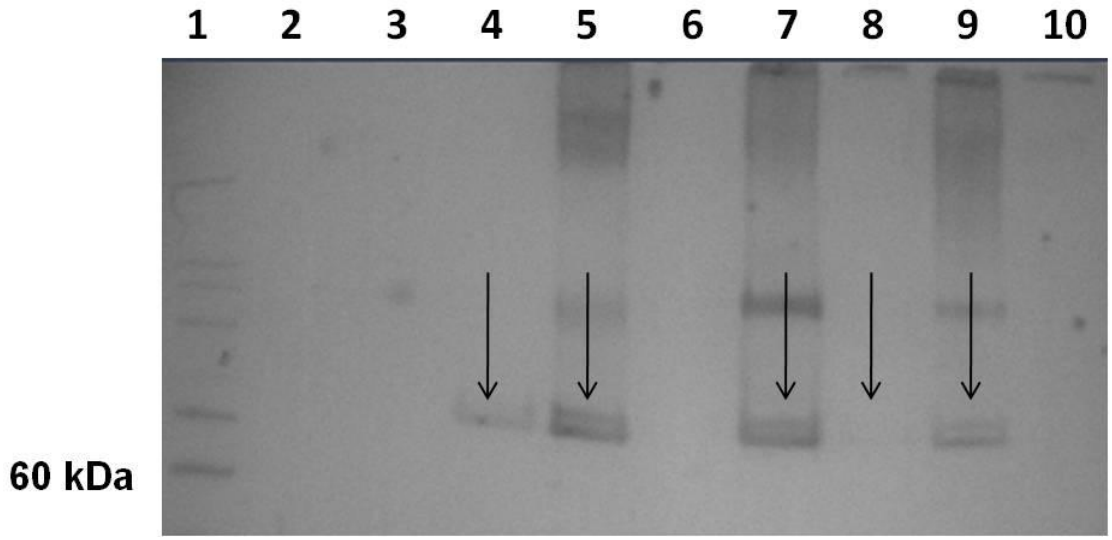


Figure 5-7 Detection of salivary amylase from saliva samples. Unstimulated saliva samples were separated using SDS PAGE before transfer to a PVDF membrane and probing with antibodies conjugated to alkaline phosphatase. Salivary amylase indicated with arrows was detected in all neat saliva samples (lane 5, 7, 9), a dilute sample (lane 8), and the 40 µg/ml positive control (lane 4). No salivary amylase was detected in the 1% (v/v) SDS negative control (lane 2) and the 100 µg/ml BSA control (lane 3). The protein ladder was present in lane 1 with sizes indicated in kDa for comparison.

5.2.7 *Protein contamination of dental forceps*

A total of 10 used and unprocessed extraction forceps and 30 decontaminated extraction forceps were provided by the CSSD department of the GDH. Forceps were transported in a sterile specimen bag to the laboratory for sampling.

The working end of each set of forceps was inserted into a 50 ml centrifuge tube containing 5 ml of 1% (v/v) SDS. Each tube was added to a boiling water bath (Grant instruments, Cambridge UK) and boiled for 10 min. A 20 µl sample was taken for quantitative analysis using the OPA assay (Section 2.3.1.3) A total of 20 samples were added together and precipitated using an Amicon® filter (Section 2.3.2.4). A further 10 samples were individually precipitated using StrataClean™ resin (Section 2.3.2.3). Precipitated samples were sampled using SDS-PAGE (Section 2.3.3). Gels were stained with silver stain (Section 2.3.4.3) and Coomassie blue (Section 2.3.4.1). The presence of protein bands was confirmed using a peak analysis by Quantity one® software (Bio-Rad version 4.6.7). Protein bands stained with Coomassie blue were sent for analysis by MS at the Glasgow University proteomics facility (Section 2.3.5). Samples were also analysed for the presence of salivary amylase and serum albumin by Western blot (Section 2.3.6).

Protein was isolated from all used unprocessed forceps (median 603 µg, range 39 – 2761 µg) and 16 decontaminated forceps (median 17, range 0 – 82) (Table 5-3). Significantly ($p<0.05$) more protein was recovered from unprocessed forceps than decontaminated forceps. For the qualitative analysis, a total of 13 protein bands representing different sized proteins were observed in all used, unprocessed samples (Figure 5-8 [a]) and 19 bands were observed when the samples were stained with SYPRO Ruby (Figure 5-8 [b], Figure 5-8 [c]). A total of 2 protein bands were observed in decontaminated forceps samples when gels are stained with silver stain (Figure 5-9 [a], Figure 5-9 [c]) and no protein was observed in 5 decontaminated (Table 2-3) forceps samples precipitated with StrataClean™ Resin (Figure 5-9 [b]). A total of 17 proteins were identified using MS (Table 5-4). Serum albumin was identified in all used, unprocessed samples (Figure 5-10 [a]) and salivary amylase was not detected in any samples (limit of

detection 20 µg/ml) (Figure 5-10 [b]). No protein could be identified from decontaminated samples.

Table 5-3 Protein isolated from used, unprocessed and decontaminated forceps samples.

Forceps	Median protein concentration/ device (µg)	Range protein concentration (µg/device)
Decontaminated (n = 30)	16.83	0 – 81.95
Unprocessed (n = 10)	603	38.5 – 2760.5

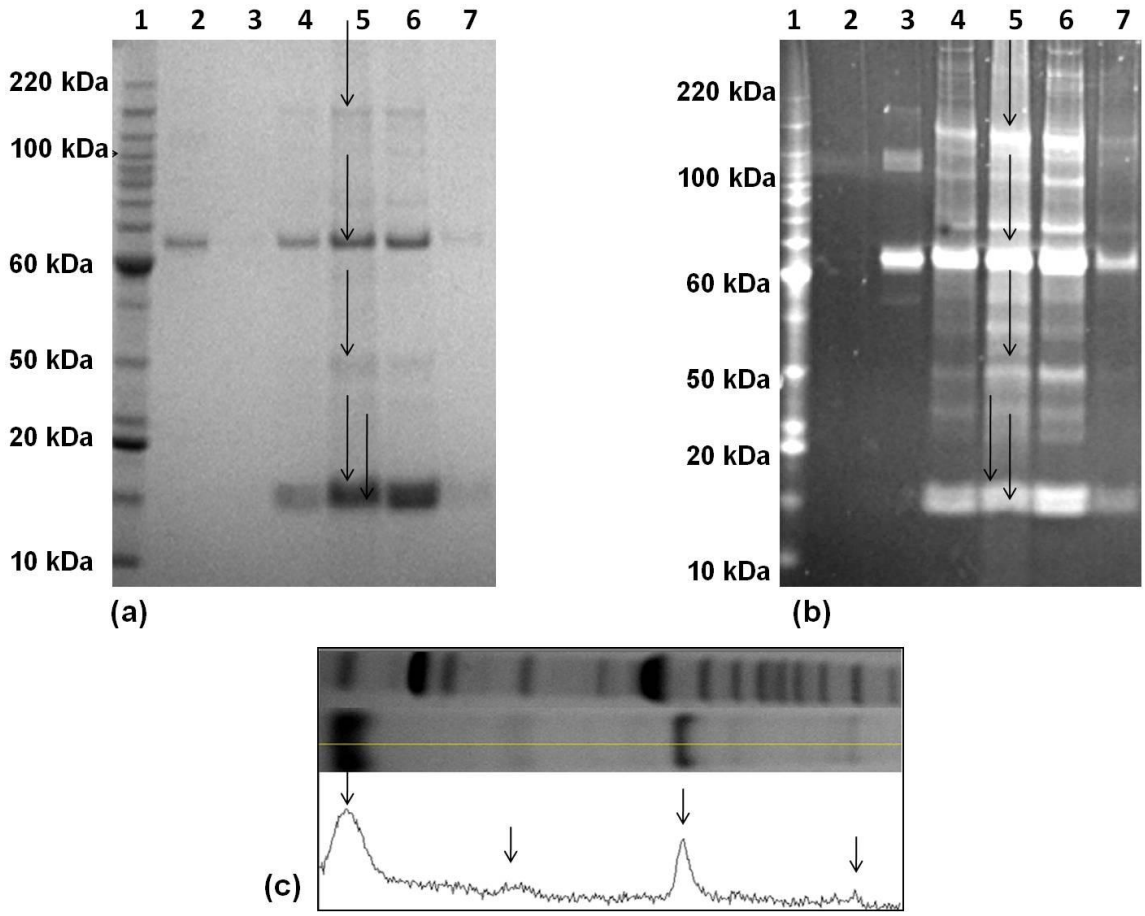


Figure 5-8 Protein content of used, unprocessed dental forceps. A sample of the eluent from 4 used, unprocessed forceps was precipitated by StrataClean™ resin and the protein content viewed by staining with coomassie blue **(a)** (lanes 4 – 7). The proteins were compared to a protein ladder (lane 1) with sizes in kDa indicated. Bands representing proteins of different sizes were found on 3 forceps samples (Lanes 4-6) **(c)**. A band of 66 kDa corresponding to 100 µg/ml BSA was observed in the positive control (lane 2) and no bands were observed in the negative control (lane 3)**.** The samples were also stained with SYPRO® Ruby **(b)** and bands were observed from each of the forceps samples (lane 4-7) and the 100 µg/ml BSA positive control (lane 3). No bands were observed in the negative control (Lane 2). Lane 1 contains the protein ladder with sizes indicated in kDa.

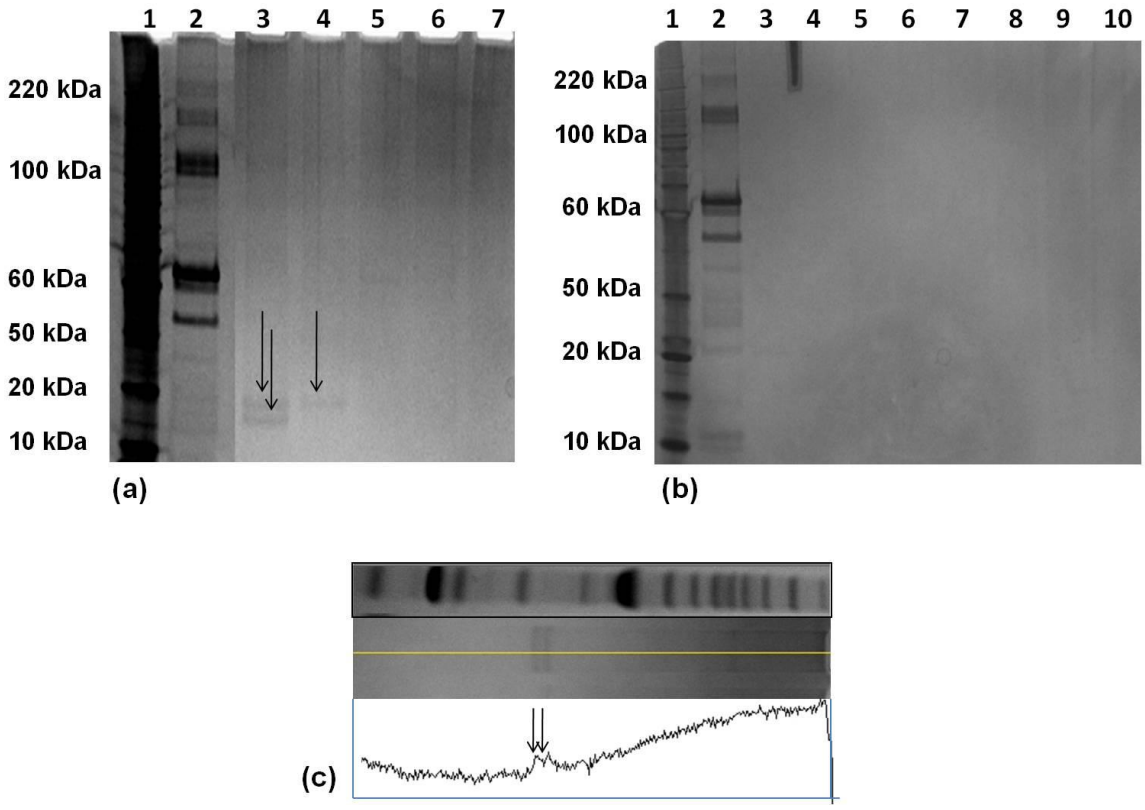


Figure 5-9 Protein contamination of decontaminated forceps. A sample of the eluent from 6 decontaminated forceps was precipitated by Amicon® filtration and the protein content viewed by staining with silver stain **(a)**. The proteins were compared to a protein ladder (lane 1) with sizes in kDa indicated. Protein bands were evident in two forceps samples (lanes 3 and 4). No protein is found in an additional 3 samples (lanes 5, 6, 7). The 100 µg/ml BSA positive control was in lane 2 and the protein ladder in lane 1 with sizes indicated in kDa for comparison. A sample of the eluent from 6 decontaminated forceps was precipitated by StrataClean™ resin and the protein content viewed by staining with silver stain **(b)**. The proteins were compared to a protein ladder (lane 1) with sizes in kDa indicated. No protein was evident in the forceps samples (lanes 5, 6, 7, 8, 9, 10). No protein was found in the negative controls (lanes 3, 4). Protein was observed in the 100 µg/ml BSA positive control (lane 2). The bands from the decontaminated forceps samples were confirmed by peak analysis **(c).**

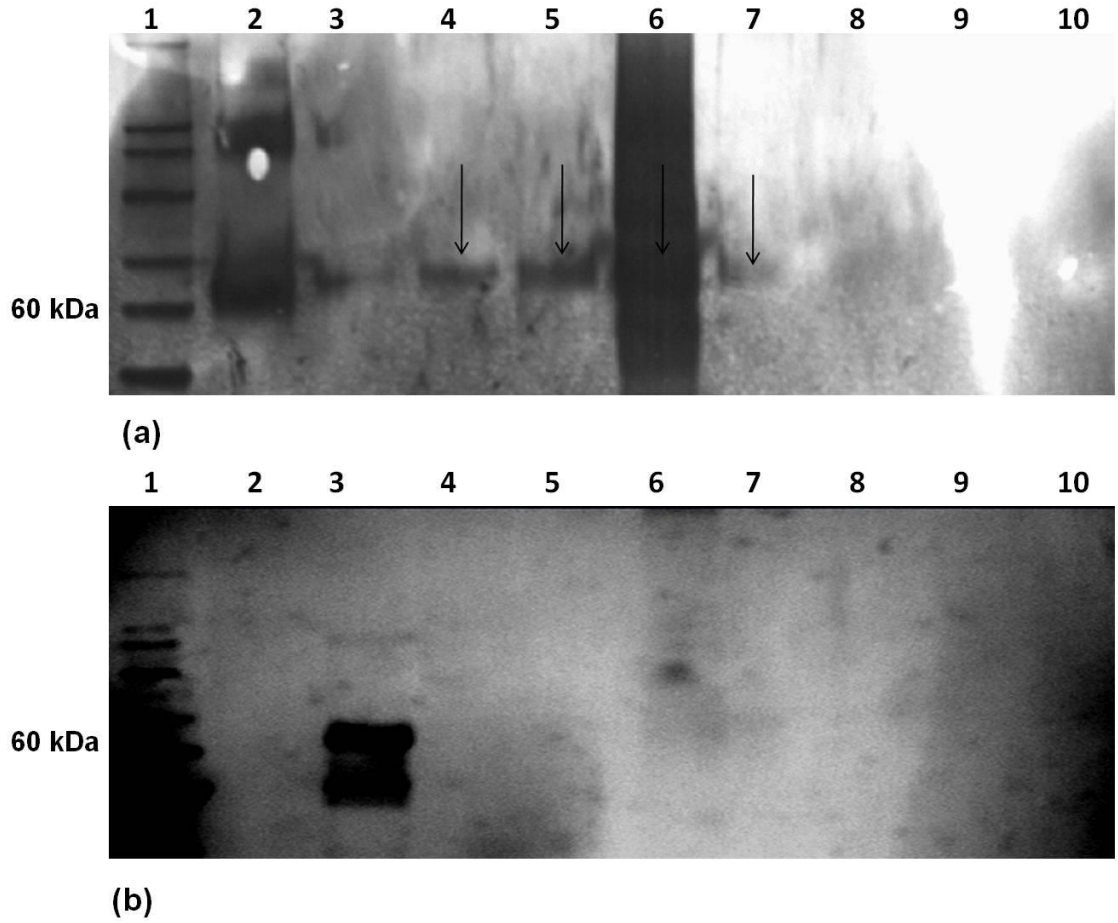


Figure 5-10 Detection of serum albumin and salivary amylase in used, unprocessed and decontaminated forceps samples. A total of 4 used, unprocessed forceps samples and 3 decontaminated forceps samples were sampled for serum albumin using probing with antibodies conjugated with alkaline phosphatase. Serum albumin **(a)** was detected in 4 used, unprocessed samples (lanes 4, 5, 6, and 7) and the 100 µg/ml BSA positive control (lane 2). No serum albumin was detected in decontaminated samples (limit of detection 0.01 µg/ml) (lanes 8, 9, 10). No salivary amylase **(b)** was detected in 4 used, unprocessed samples (lanes 4, 5, 6, and 7) or the decontaminated samples (lanes 8, 9, 10) (limit of detection 20 µg/ml). Salivary amylase was detected in the 40 µg/ml positive control (lane 3).

Table 5-4 Protein identified from used, unprocessed dental forceps

Protein recovered	**Peptide size (kDa)**
α- globin	**11**
Haemoglobin α 1-2 hybrid	**11.5**
Haemoglobin chain A	**15**
β – globin	**15.2**
Unnamed protein product (Homo sapiens) (x2)	**15.8 – 69**
Haemoglobin β chain	**15.8**
Haemoglobin δ	**15.8**
Haemoglobin chain B	**18**
Immunoglobulin heavy chain	**18.2**
Immunoglobulin light chain	**23.2**
Carbonic anhydrase	**28**
Pasteurella multocida OMPH / hypothetical protein PM0786	**36 (peptide 14.6)/ 38 (peptide 12)**
Cytokeratin	**62**
Serum albumin	**67**
Serum albumin precursor	**69**
Serotransferrin	**77**
Transferrin	**80**

5.2.8 *Protein contamination of dental handpieces*

A total of 30 W&H TA-98 high speed HP's, 30 W&H WA-56 low speed HP's, and 25 W&H S11 surgical HP's were provided by the GDH CSSD department before decontamination (Table 2-3). In addition, a total of 20 W&H TA-98 high speed HP's, 20 W&H WA-56 low speed HP's and 10 W&H S11 surgical were provided after decontamination (Table 2-3). Each HP was processed as detailed in Section 2.1.2. Each HP turbine was immersed in 2 ml of 1% (v/v) SDS, each spray channel was immersed in 8 ml of 1% (v/v) SDS and each surgical gear was immersed in 15 ml of 1% (v/v) SDS. Each tube was added to a boiling water bath (Grant instruments, Cambridge, UK) and boiled for 10 min. A 20 µl sample was taken for quantitative analysis using the OPA assay (Section 2.3.1.3). For used unprocessed HP's the eluents of 20 high speed turbines, 20 low speed spray channels and 15 surgical gears were added together and precipitated using Amicon® filters (Section 2.3.2.4). The retentate of each HP part was kept for analysis. For decontaminated HP's, the eluents from 20 high speed turbines, 20 low speed spray channels and 10 surgical gears were precipitated using Amicon® filters (Section 2.3.2.4). Precipitated samples were sampled using SDS-PAGE (Section 2.3.3). Gels were stained with silver stain (Section 2.3.4.3) and Coomassie blue (Section 2.3.4.1). The presence of protein bands was confirmed using a peak analysis by Quantity One® software (Bio-Rad version 4.6.7). Protein bands stained with Coomassie blue were sent for analysis by MS at the Glasgow University proteomics facility (Section 2.3.5). Samples were also analysed for the presence of salivary amylase and serum albumin by Western blot (Section 2.3.6).

Protein contamination was isolated from 17/30 high-speed turbines, 22/30 of low speed spray channels, and 20/20 of surgical S11 gears (Table 5-5, Figure 5.11). Before decontamination, the surgical gear contained significantly ($p < 0.001$) more protein than the high speed turbines and low speed spray channels. No significant difference was observed between protein isolated from low speed spray channels and high speed turbines. After decontamination (Table 2-3), protein was significantly ($p<0.001$) reduced in surgical gears and spray channels ($p<0.05$) (Table 5-6, Figure 5-11). After precipitation of samples, protein and mucopolysaccharide bands were observed in each used unprocessed

instruments (Figure 5-12[a][b][c],Figure 5-13) and decontamined surgical gears, (Figure 5-14 [a][b][c]).For Western blot analysis,salivary amylase was not detected in any used, unprocessed and decontaminated sample (limit of detection 20 µg/ml) (Figure 5-15). Serum albumin and salivary mucin 5b was identified in the used, unprocessed surgical HP gears but not in the high speed turbines or low speed spray channels (Figure 5-16, Figure 5-17). An ELISA was conducted on all HP samples using a rabbit anti- human salivary amylase antibody as the capture antibody, a sheep anti-human salivary amylase antibody as the secondary antibody and a goat anti-rabbit IgG antibody conjugated with horseradish peroxidise was used as the detection antibody. Salivary amylase was detected in all used, unprocessed samples (Table 5-7) and no salivary amylase was detected in decontaminated samples (Table 5-7). A total of 11 proteins were identified by MS (Table 5-8).

Table 5-5 Protein contamination of used, unprocessed HP's.

Handpiece part	Median protein concentration (µg/device)	Range protein concentration (µg/device)	Precipitated concentration (µg/ml)
High speed turbine (n = 30)	1.3	<5 – 210	45.6
Low speed spray channel (n = 30)	15.41	<5– 55.4	31.8
Surgical gear (n=25)	350	127.5 – 1936	194.8

Precipitated samples consisted of 20 high speed turbines eluents, 20 low speed spray channel eluents, and 15 surgical gear eluents.

Table 5-6 Protein contamination of decontaminated HP's.

Handpiece Part	Median protein concentration (µg/device)	Mean protein concentration (µg/device)	Range protein concentration (µg/device)	Precipitated concentration (µg/ml)
High speed turbine (n = 20)	0	6.4	<5– 18	0.96
Low speed spray channel (n = 20)	0	11.1	<5 – 103	10.6
Surgical gear (n=10)	0	3.2	<5 – 64.5	51

Precipitated samples consisted of 20 high speed turbines eluents, 20 low speed spray channel eluents, and 15 surgical gear eluents.

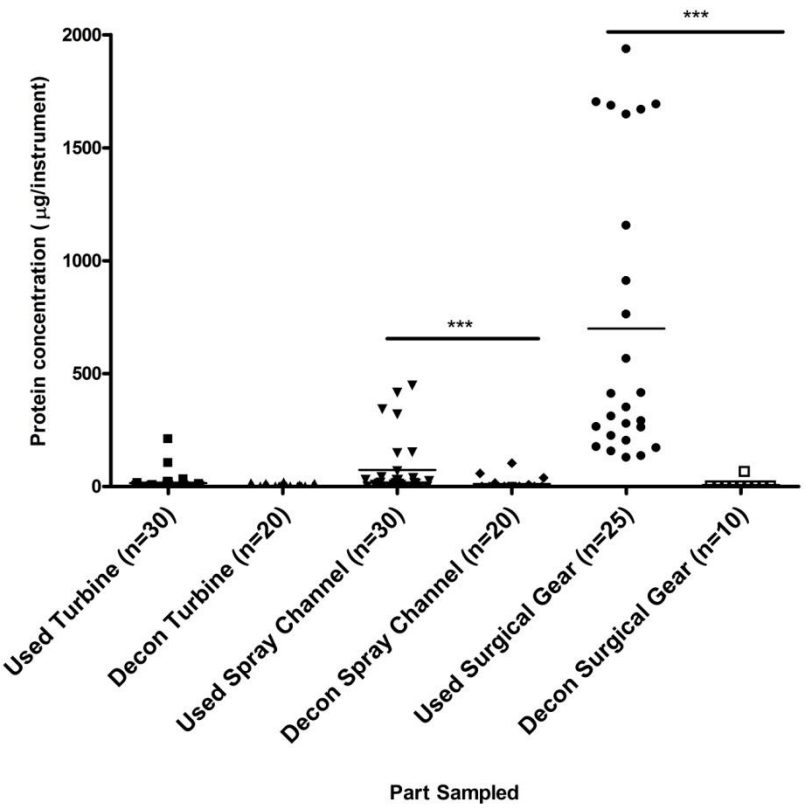


Figure 5-11 Quantitative protein analysis of dental HP parts. Internal HP parts were sampled for protein before and after decontamination. The turbine of the high speed HP, the spray channel of the low speed HP and the gear of the surgical HP were sampled. Before decontamination, surgical HP gears contained the most protein with a median of 350 µg. This was significantly (***=$p<0.001$) reduced after decontamination to a median of <5 µg. A median of 1.3 µg of protein was isolated from high speed turbines before decontamination and reduced to a median of 0 µg after decontamination. For spray channels, a median of 15.41 µg of protein was isolated before decontamination which was significantly ($p<0.05$) reduced to 0 µg of protein after decontamination.

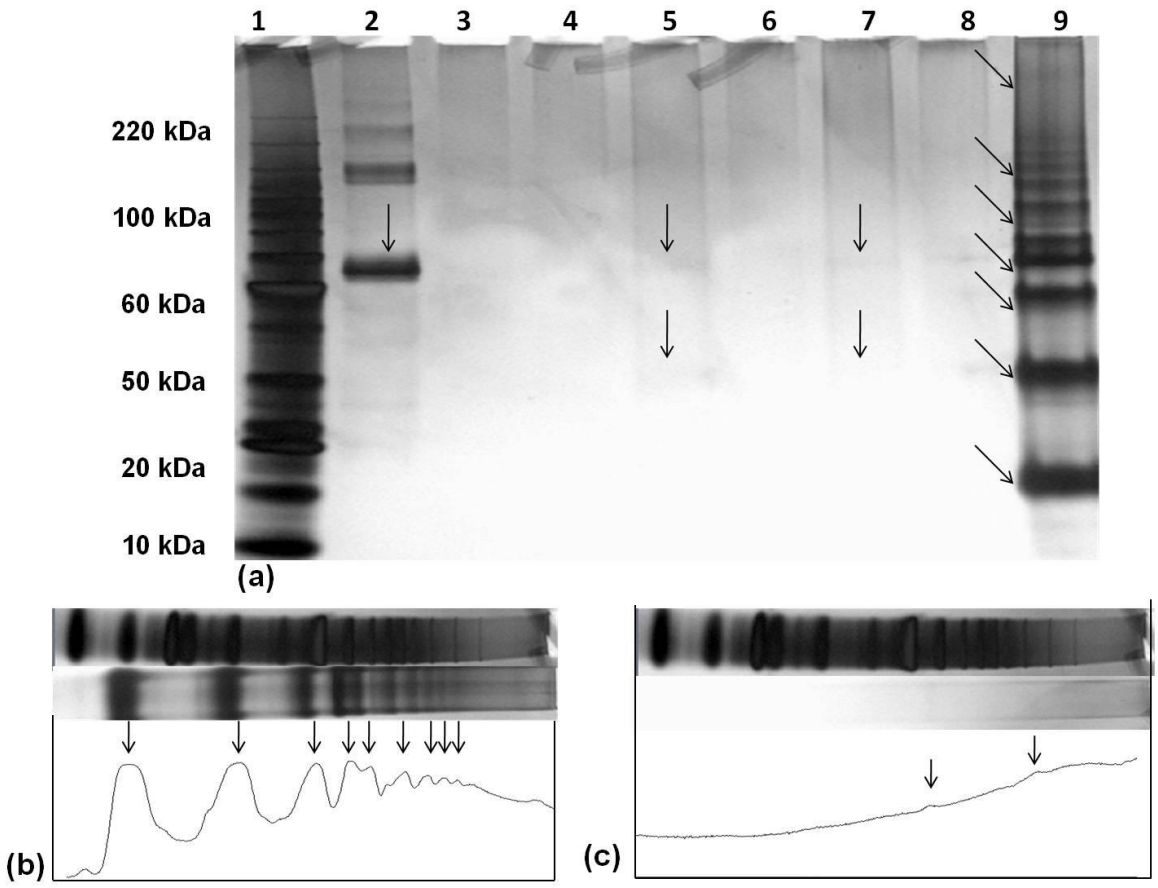


Figure 5-12 Protein contamination of used, unprocessed HP's. Eluents from used, unprocessed turbines, low speed spray channels, and surgical gears were precipitated together using an amicon filter and the gel stained with silver stain. Protein was observed in the high speed turbine samples with 3 distinct bands present (lane 5), **(**the low speed spray channel samples with 2 distinct bands present (lane 7), and the surgical gears where 9 distinct bands were present (lane 9) **(a)**. No protein was observed in the negative controls (lane 3, 4, 6, 8). Lane 2 consisted of the 100 µg/ml BSA positive control and lane 1 contained the protein ladder with the sizes in kDa for comparison. The bands from the surgical gears **(b)** and the turbines **(c)** were confirmed with peak analysis.

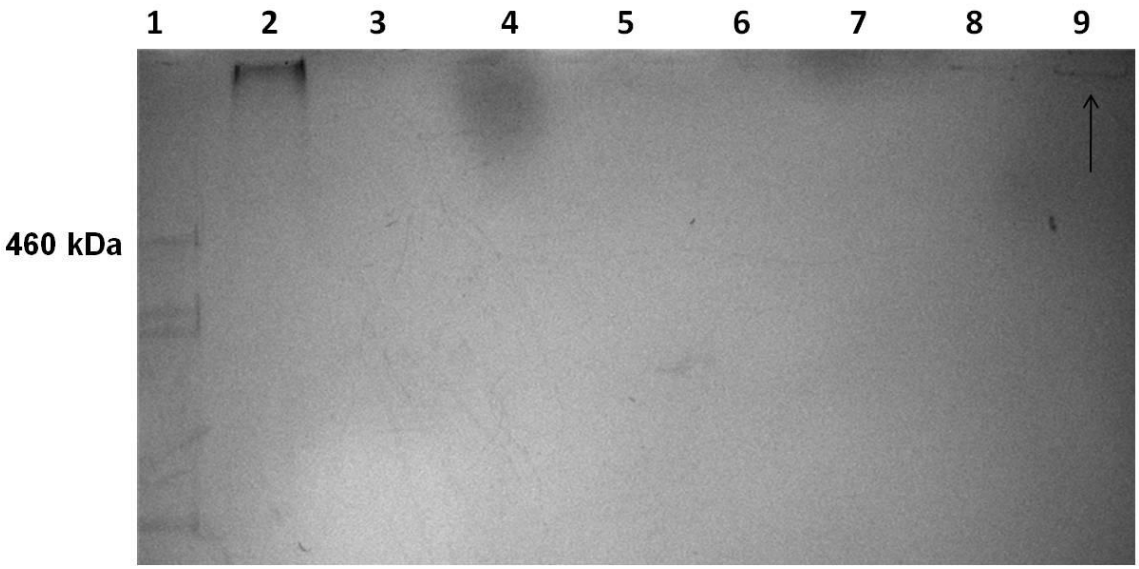


Figure 5-13 PAS staining for mucopolysachharides in dental HP's. Eluents from used, unprocessed turbines, low speed spray channels, and surgical gears were precipitated together using an amicon filter and the gel stained with PAS reagent. Mucopolysaccharide was detected in the surgical HP gear (lane 9) and the 100 µg/ml salivary mucin control (lane 2). No protein was detected in the negative controls (lanes, 3,4,5,6) or the high speed HP turbines (lane 7) and the low speed HP spray channels (lane 8). The Himark™ protein ladder was included for size comparison (lane 1).

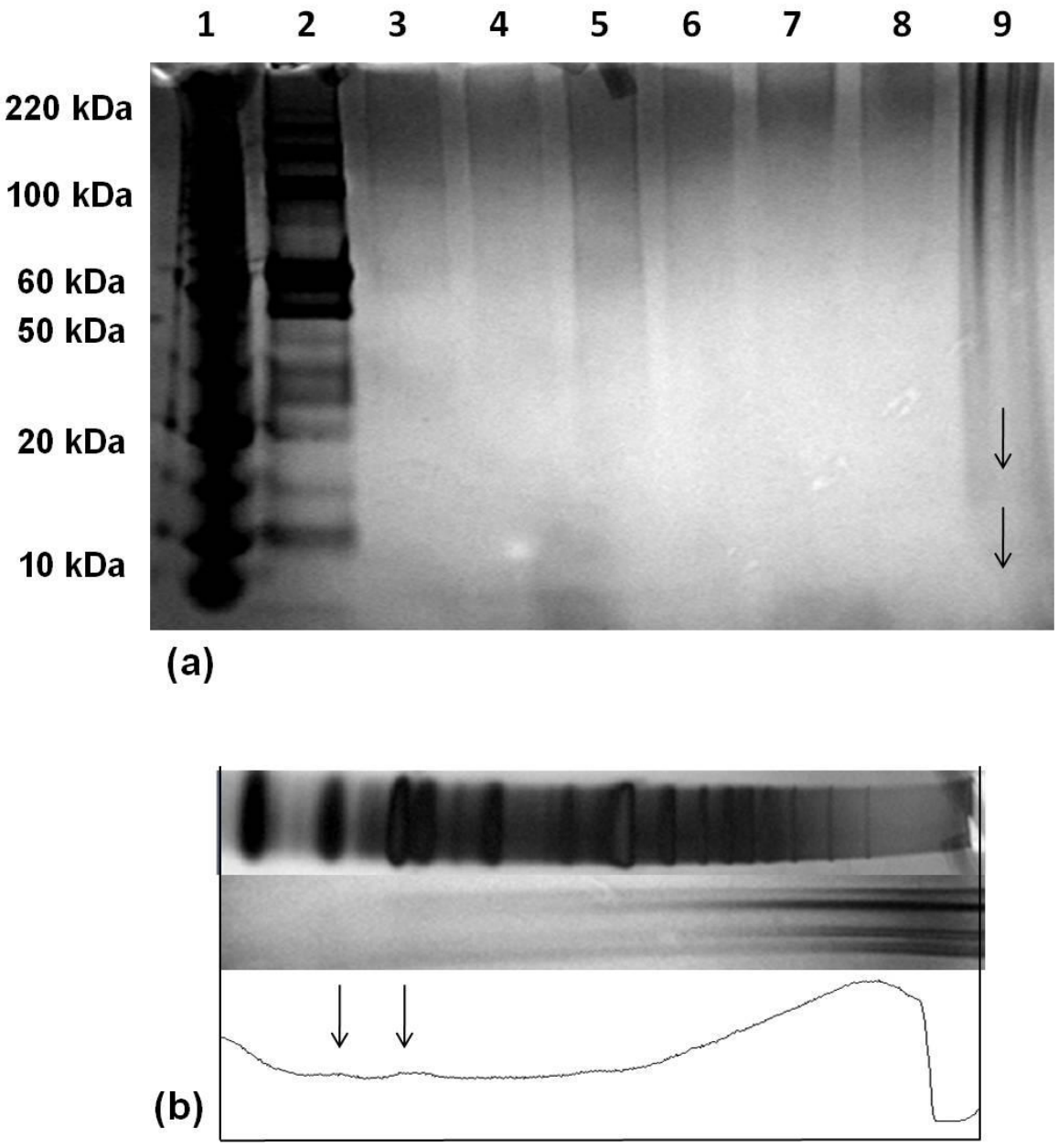


Figure 5-14 Protein contamination of decontaminated dental HP's. Eluents from decontaminated turbines, low speed spray channels, and surgical gears were precipitated together using an amicon filter. Protein was observed in the surgical gears (lane 9) with 2 distinct bands visible **(b).** No protein bands were observed in the high speed turbines (lane 5), spray channels (lane 7) and or the negative controls (lane 3, 4, 6, 8). Lane 2 consisted of the BSA positive control and lane 1 contained the protein ladder with the sizes in kDa for comparison.

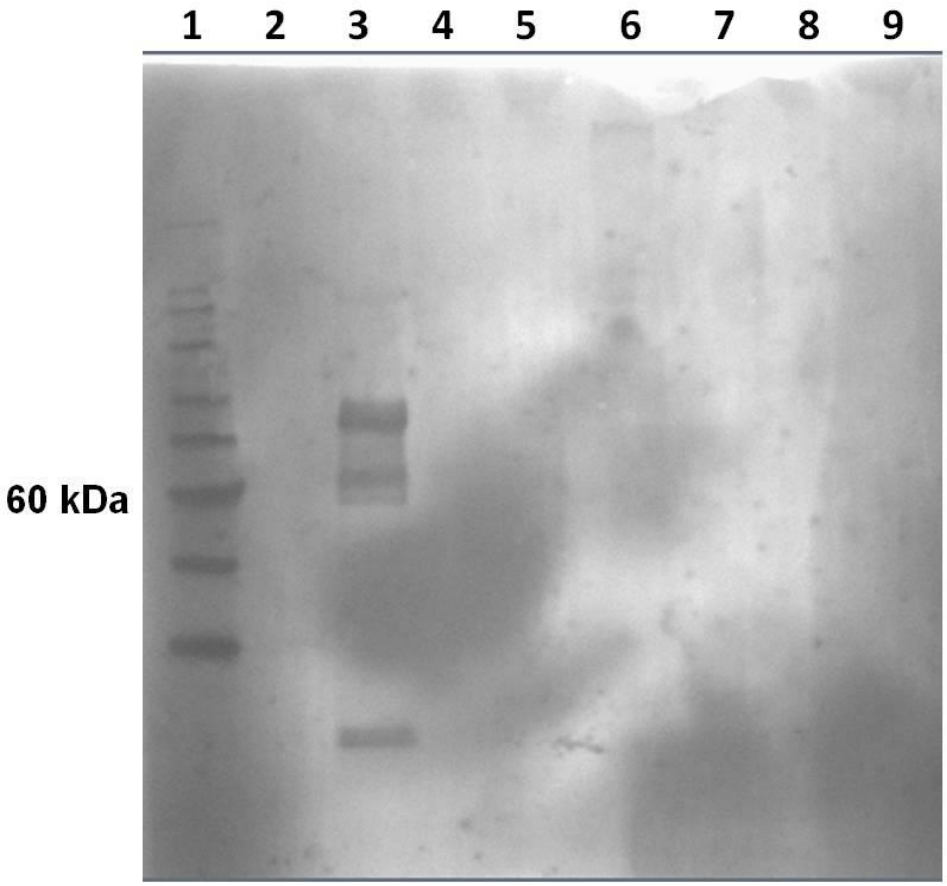


Figure 5-15 Detection of salivary amylase in used, unprocessed HP samples. No salivary amylase (limit of detection 20 µg/ml) was detected in the high speed turbine samples (lane 8), the low speed spray channel samples (lane 9) and the surgical gear samples (lane 10), or the negative controls (lanes 2, 4, 5, 6). Salivary amylase was observed in the salivary amylase positive control (lane 3). Lane 1 contains the protein ladder with sizes in kDa indicated for size comparison.

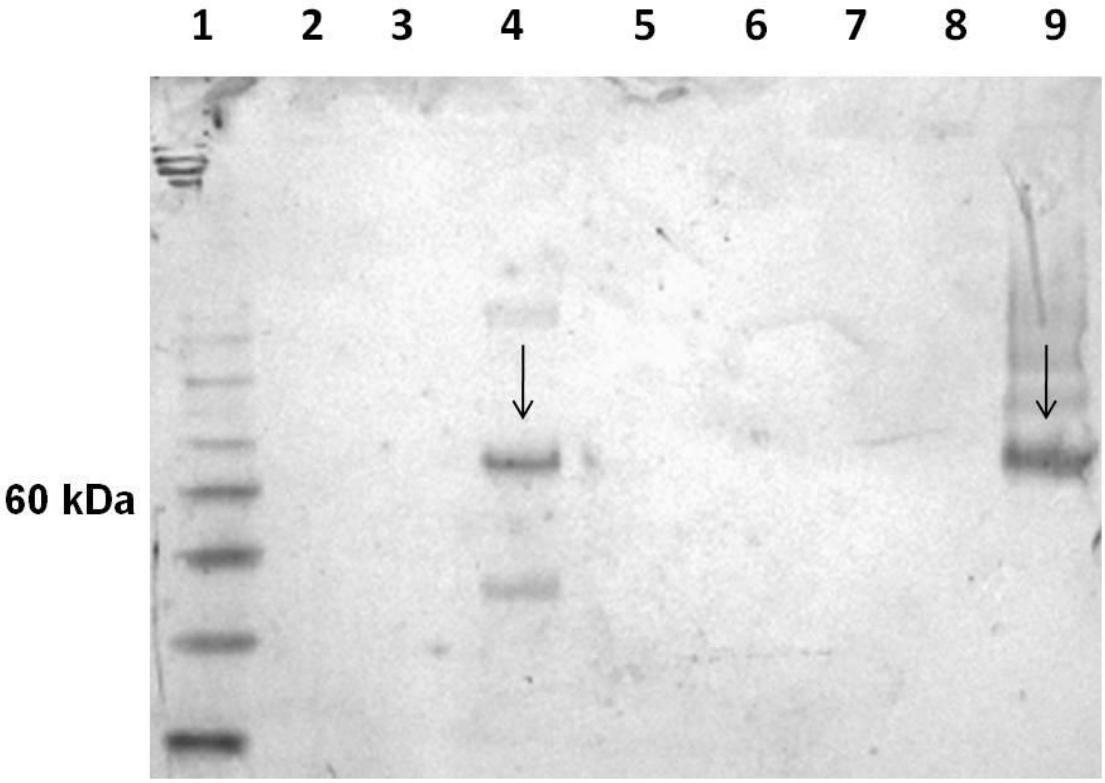


Figure 5-16 Detection of serum albumin in used, unprocessed handpiece samples. Serum albumin was detected in the surgical gear samples (lane 9) and the 100 µg/ml serum albumin positive control (lane 4). No serum albumin was detected (sensitivity 0.01 µg/ml) in the negative controls (lanes 2, 3, 5, 6), the high speed turbine samples (lane 7), or the low speed spray channel samples (lane 8). Lane 1 contains the protein ladder for size comparison.

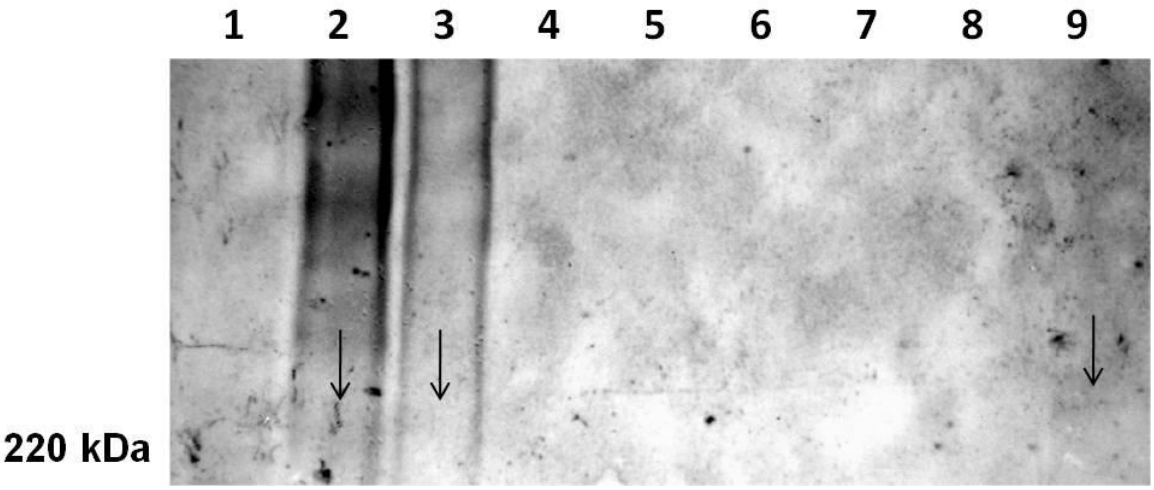


Figure 5-17 Detection of salivary mucin in used, unprocessed handpiece samples. Salivary mucin 5b was detected in the surgical gear samples (lane 9) and the saliva controls (lanes 2, 3). No salivary mucin (limit of detection 1/10 dilution of saliva) was detected in the negative controls (lanes 4, 6, 8), the high speed turbine samples (lane 5), or the low speed spray channel samples (lane 7). Lane 1 contains the protein ladder for size comparison.

Table 5-7 Salivary amylase concentration detected using ELISA in precipitated used, unprocessed and decontaminated HP samples.

Sample	State	Salivary amylase concentration detected (µg / ml)
Turbine	Used, unprocessed	1.8
	Decontaminated	0
Spray	Used, unprocessed	2.9
	Decontaminated	0
Surgical	Used unprocessed	6.1
	Decontaminated	0

Table 5-8 Proteins identified from used, unprocessed surgical handpiece samples.

Protein recovered	Peptide size (kDa)
50S Ribosomal protein	11.5
Alpha globin	13.5
Haemoglobin chain D	15.8
Haemoglobin subunit beta	15.8
Haemoglobin subunit ε	15.8
Haemoglobin beta chain	15.8
Haemoglobin beta	15.8
Haemoglobin chain B	18
Serum albumin	67
Hypothetical protein (Homo sapiens)	69
Conserved hypothetical protein *Paracoccidiodes brasilineus*	119

5.3 Discussion

A qualitative analysis of instrument protein contamination is essential to determine cross infection risks and to help inform the development of cleaning processes and chemicals. This study presents validation work on the extraction and sampling of protein contamination and utilises a method for the qualitative analysis of instrument protein contamination. This study has added advantage over previous investigations that have sacrificed instruments during protein extraction (Baxter et al., 2006). In contrast, this study involved taking HP's directly from the GDH supply chain, thus minimising cost and unnecessary waste.

Previous work into the analysis of instrument residual protein contamination has been mostly limited to quantitative studies (Smith et al., 2005, Murdoch et al., 2006, Baxter et al., 2006, Vassey et al., 2011) . Studies are concerned with the validation and monitoring of cleaning processes as protein is used as a marker of cleanliness. Smith *et al*. (2005) studied the protein contamination of endodontic files decontaminated in dental practice using different methods for cleaning. Cleaning files using manual washing alone resulted in a median of 4.9 µg of protein present, and cleaning with a manual wash and ultrasonic bath resulted in a median of 5.6 µg of protein present (Smith et al., 2005). For dental instruments, a survey of the efficacy of washer disinfectors and ultrasonic baths on cleaning various dental instruments found protein on instruments before and after the decontamination process with a median of 5.75 µg of protein isolated after cleaning using a washer disinfector (Vassey et al., 2011). A study by Murdoch et al. (2006) on the protein contamination of hospital decontaminated instrument trays showed that 17% of instruments sampled had protein concentrations above an arbitrary cut off value of 200 µg with one instrument containing 45 mg of protein. A similar study on instrument trays was carried out by Baxter *et al*. (2006) whom isolated a median of 267 µg/instrument from 5 instrument trays containing a total of 120 instruments.

These studies all state in their conclusions that the presence of protein may constitute a cross contamination risk (Smith et al., 2005, Baxter et al., 2006). The only protein that is capable of causing disease is the prion protein, the causative agent of CJD (Arakawa et al., 1991) and all the aforementioned studies

mention CJD as the driving force behind the measurement of protein contamination (Smith et al., 2005). Whilst a quantitative analysis of protein contamination can give an indication of contamination, it is impossible to put these protein concentrations into context without a qualitative analysis of protein contamination.

Studies into qualitative analysis of instrument protein have been conducted on ophthalmic phacoemulsification tips (Nuyts et al., 1999) and orthodontic bands (Benson and Douglas, 2007). *Nuyts et al*. (1999) utilised Coomassie blue for general staining and Western Blot for specific lens proteins to validate cleaning procedures. No lens proteins were detected even if no cleaning procedure was applied (Nuyts et al., 1999). Whilst the sensitivity of detection was calculated as 48 ng, the sensitivity of the extraction method was not stated and this may be the reason for the lack of protein isolated. A study into the presence of serum albumin and salivary protein on orthodontic bands found that each protein was found on 50% of bands after cleaning (Benson and Douglas, 2007). This study used ELISA though no quantitative data was measured (Benson and Douglas, 2007). The application of some of the aforementioned methods, in conjunction with quantitative analysis (Chapter 3), can build a complete picture of instrument protein contamination.

For all validation work a total of 3 proteins were used for the validation of coomassie blue and silver staining. BSA was used as a model protein representing serum albumin, the most abundant protein in blood which is a common contaminant of the instruments sampled (Desroches et al., 2007), salivary amylase as the most abundant protein in human saliva (Hu et al., 2007a), and salivary mucin has a glycosylated structure containing mostly sugars that is abundant in the oral cavity (Liu et al., 1998). Only salivary mucin was used for the validation of PAS staining as this method stains sugars which are not abundant on BSA and salivary amylase proteins (Ramasubbu et al., 1996, Sugio et al., 1999). To determine the sensitivity of salivary mucin Western blotting, human saliva was used due to the unavailability of purified human salivary mucin protein.

For protein desorption, the solutions and processes used were initially selected for their use in previous studies (Smith et al., 2005), recommendations by BS—EN ISO-15883 part 1, and for the lack of corrosive properties. Some protein removal solutions may also denature the protein and not allow visualisation of proteins by SDS PAGE. The protein not recovered may remain on the surface or may be due to protein denaturation not allowing measurement. The most efficacious method for protein recovery sampled, through measurement of the OPA assay, was boiling in 1% (v/v) SDS validated by SDS PAGE and the OPA assay. SDS is an anionoic surfactant that is recommended by the BS-EN-ISO-15883 part 1 for desorption of protein from a test piece surface. SDS denatures the secondary and tertiary structure of proteins forming the proteins into rod shapes (Weber and Kuter, 1971). A molecule of SDS can associate with the peptide backbone of the protein and applies a negative charge, the strength of which is based on the protein size (Weber and Osborn, 1969). The boiling of the SDS solution increases binding to the protein molecule, which may account for the increase in removal when compared to sonication. A solution of 0.1M NaOH was sampled due to its application for the inactivation of prion proteins (Bauman et al., 2006). A solution of NaOH is an alkaline solution that causes hydrolysis of the proteins and reverses the interaction of the protein adsorbed to the stainless steel surface (Bauman et al., 2006, Desroches et al., 2007). This occurs through the alteration of the electrostatic charge of the protein residues and reduces the electrostatic attraction of the surface (Sakiyama et al., 2004). A solution of 0.1M NaOH was found to cause corrosion of HP turbines showing the unsuitability for HP sampling. Decon®90 is also an alkaline detergent with a pH of 10.4 which has been used for the desorption of protein in previous instrument contamination studies (Smith et al., 2005). The Decon®90 detergent may have a similar mode of action to the sodium hydroxide though the exact composition of the detergent is unknown. The quantitative data was combined with SDS-PAGE to visualise the protein recovered as the OPA assay relies on the reaction with primary amines, a denatured protein may still cause a positive reaction if parts of the structure of are still intact (Roth, 1971). Each gel showed that BSA fraction V was isolated with each cleaning solution though 0.1M NaOH was not sampled using SDS-PAGE due to the aforementioned corrosion of turbines.

Staining of gels was undertaken by coomassie blue, Silverquest® silver stain, and SYPRO® Ruby staining representing an organic dye, a silver stain and a fluorescent stain. The sulfonic groups of Coomassie blue dye binds to the protein molecule by electrostatic and hydrophobic binding to basic amino acids such as lysine, arginine and histidine residues in the protein (Compton and Jones, 1985, Congdon et al., 1993). Coomassie blue stain has a reported sensitivity of 0.5 µg of protein (Candiano et al., 2004) which is comparable to the 0.2 µg of protein found in the validation work. Whilst the insensitivity of Coomassie blue staining may result in an incomplete picture of the protein contamination, the reversible interactions allow compatibility for further downstream protein identification mass spectrometry (Candiano et al., 2004). Use of the Silverquest® silver stain results in an increased sensitivity of protein detection to less than 0.02 ng of protein which is comparable to previous results (Rabilloud, 1992). The staining is due to the reaction of silver ions to metallic silver by the protein molecule in the presence of formaldehyde. The silver staining kit uses less formaldehyde than other silver staining techniques to enable MS compatibility though the low concentration of proteins did not allow identification of silver stained bands by mass spectrometry. SYPRO ruby is a ruthenium based fluorescent stain that has reported sensitivities comparable to silver staining. The stain binds to lysine, arginine, and histidine residues and can be visualised using ultraviolet light (Lopez et al., 2000), though this is not the optimum method for visualisation.

Precipitation of samples was necessary due to the comparatively large sample volumes and smaller protein concentrations compared to classical proteomic studies and other elements in the sample such as lubricating oil or metallic fragments that may affect the purity of the sample (Aguilar et al., 1999). Initial work with non-precipitated HP samples did not reveal any protein bands despite positive reactions with the OPA assay. The efficacy of each method was assessed using a volume of protein solution used for immersion of a high-speed turbine and a protein concentration based on a value that has been isolated from used, unprocessed HP's. A total of 4 precipitation methods were assessed each with different mechanisms. Amicon® ultra centrifugal filters were found to recover the most protein of the methods sampled. Previous studies into the efficiency of the protein precipitation methods have shown that precipitation with TCA precipitation recovering 24% of protein and acetone precipitation recovering 69%

of rat brain protein (Fic et al., 2010). Whilst the TCA precipitation efficiency was similar to the value in this study, the acetone precipitation recovery was more than was recovered in this study. This may be due to the increased starting protein concentration or the increased number of proteins in the samples as each precipitation technique has different effects on different proteins (Zellner et al., 2005). For ultracentrifugation methods, the filters trap proteins with a size larger than 15 kDa in a filter in a smaller volume of solution, StrataClean™ resin acts through direct interaction of the resin with protein hydroxyl groups whilst acetone precipitation causes aggregation of proteins through dehydration of any water molecules (Simpson and Beynon, 2009) and TCA exposure results in the unfolding of the protein through disruption of the electrostatic interactions by the trichloroactetate ions (Sivaraman et al., 1997). Filtration was the only method sampled that does not result in protein binding or modification of protein structure for downstream identification by mass spectrometry and coupled with the comparatively high rate of recovery was ideal for processing of HP samples (Jiang et al., 2004, Simpson and Beynon, 2009).

Dental extraction forceps were used as a model instrument to test the quantitative and qualitative methods for measuring protein contamination due to many factors. The forceps are directly exposed to blood and saliva contamination during routine use which indicate that more protein will be present than the dental HP, and the working end of forceps contains a rough surface structure that may trap protein and inhibit removal by cleaning processes (Smith et al., 2005). The forceps also have direct access to contamination and do not require dismantling. Previous studies have detected residual protein on decontaminated forceps with a median of 17 µg/instrument and a range of 0 – 213 µg/instrument (Murdoch et al., 2006). Work by Vassey *et al*. (2011) into used, unprocessed and decontaminated dental instruments from dental practices found a median of 462 µg which was reduced to 27 µg of protein upon cleaning using an AWD (Vassey et al., 2011). These values are comparable to those found in this study. These studies did not attempt to identify these proteins using qualitative analysis. In this study, a total of 16 protein contaminants of used, unprocessed dental extraction forceps were identified using mass spectrometry and serum albumin was detected using Western blot. The majority of identified proteins were associated with human blood or saliva

including serum albumin, carbonic anhydrase and haemoglobin chains and proteins from *P. multocida* which indicate the presence of bacteria (Rose and Mathai, 1977, Hortin, 2006, Hu et al., 2007a). Western blot, with a sensitivity of 0.02 µg of amylase protein, did not detect salivary amylase from forceps samples before or after decontamination. Studies have shown that whilst amylase is the most abundant protein in saliva (Hu et al., 2007a), it is present at concentrations of 20 µg/ml of saliva (Sivakumar et al., 2009). This is lower than the positive control of the sample used which was 40 µg/ml from a purified solution and the amount of saliva coated on forceps may contain a lower level of amylase than the limit of detection of the assay. Use of more sensitive chemoluminescence Western blot detection methods may allow the detection of lower concentrations of amylase due to the increased sensitivity of detection associated with this method compared to alkaline phosphatase (Falk and Elliott, 1985). The ELISA method has previously been utilised to detect salivary amylase from orthodontic bands and is more sensitive than the Western blot method which may indicate that amylase could be detected using these methods (Benson and Douglas, 2007). Protein was detected in two decontaminated forceps samples without visible blood contamination when silver stain was applied but the low concentration did not allow identification by mass spectrometry. Comparison to the protein bands found in used, unprocessed samples show the proteins in the size range are carbonic anhydrase and the outer membrane proteins of *P. multocida,* though it is not possible to conclude that these are the remaining proteins without further tests.

This was the first study to examine residual protein contamination of dental HP's where prior research has primarily focused on microbial contamination (Herd et al., 2007, Kellett and Holbrook, 1980). The complexity of the internal HP structure has focused attention on the efficacy of the HP cleaning process and the ability to remove protein from internal locations associated with microbial contamination. Residual protein was detected in each HP part with the largest amount of protein being isolated from the surgical HP gear, which has the largest surface area and is used for more invasive procedures. The lowest amount of protein was isolated from turbines, which have the smallest surface area. Protein contamination was also evident in SDS PAGE gels after sample pooling though protein could only be detected from turbines and spray channels

when silver stain is applied. In common with the extraction forceps, the surgical HP gears contained several bands representing different sized blood proteins of detectable concentration with serum albumin being detected in this sample by Western blot. Serum albumin was also detected using MS in addition to 8 blood proteins, a fungal protein and a ribosomal protein. No salivary amylase was detected in any sample using Western blot though the ELISA reaction showed the presence of a small concentration of salivary amylase in all used, unprocessed samples.

In conclusion, the methods highlighted can be utilised to undertake a quantitative and qualitative analysis of instrument protein contamination and fill a large gap in instrument contamination knowledge. The main weakness of the qualitative analysis, mainly the inability to identify small concentrations of protein, must be overcome as any contaminants left after decontamination may present a cross infection risk. The precipitation of more instrument eluent to increase the concentration of common contaminants or the application of more sensitive proteomic identification techniques may overcome these shortcomings. The technique relies on the desorption of protein from the surface and is only as sensitive to how much can be removed from the surface. This work should be combined with surface analysis of instruments to construct a complete picture of instrument contamination before and after the decontamination process. The methods in combination can also validate different instrument cleaning processes and highlight any improvements that need to be made in processes or cleaning solutions if certain proteins remain after decontamination.

6 Chapter 6: Surface Analysis of Dental Handpiece Parts.

6.1 Introduction

The inspection stage of the instrument decontamination process is essential to detect any failures of the cleaning process which is visible soil that will not be removed by subsequent sterilization and may also inhibit the sterilization process (Amaha and Sakaguchi, 1954, Lipscomb et al., 2008) . The current method utilised routinely in SSD's relies on a visual inspection to ensure that the instrument is not damaged and to detect any contaminants that are visible to sight including haemoglobin in blood (Lipscomb et al., 2008). Previous research into instrument contamination has also utilised visual analysis by illumination and magnification and also relied on desorption of contamination from the surface through the application of surface swabbing or flushing of the instrument for the removal of contaminants from the instrument surface for analysis (Smith et al., 2005, Herd et al., 2007). These visual and desorption methods are also recommended by the European standard BS-EN-ISO-15883 part 1 to validate the efficacy of cleaning processes. Concerns have been raised at the sensitivity and limits of detection of both methods (Lipscomb et al., 2006b, Baxter et al., 2006), and of the contamination that remains on the surface of the instrument during sampling that would not be detected. The contaminants observed will also depend on the analysis being performed on the instrument samples whilst other residual contamination may get overlooked.

In the previous chapters of this study, identification of contaminants has relied on desorption of the contaminants from the surface. This has a calculated efficacy of 67% of bacteria removed (Chapter 4) and 87% of protein (Chapter 5). The remaining contaminants on the surface require analysis to build a complete picture of instrument contamination. Surface analysis techniques are increasingly being utilised to provide a microscopic view of instrument surfaces (Baxter et al., 2006). These techniques can visualise bacterial cells and other tissue debris whilst methods also exist that allow for the identification of these contaminants at the elemental level (Lipscomb et al., 2006b). Other techniques allow for a quantitative analysis of any biological material present on the surface without the need for the removal of contamination (Baxter et al., 2006), or the treatment of the instrument with any chemicals that may cause damage to sampled instruments.

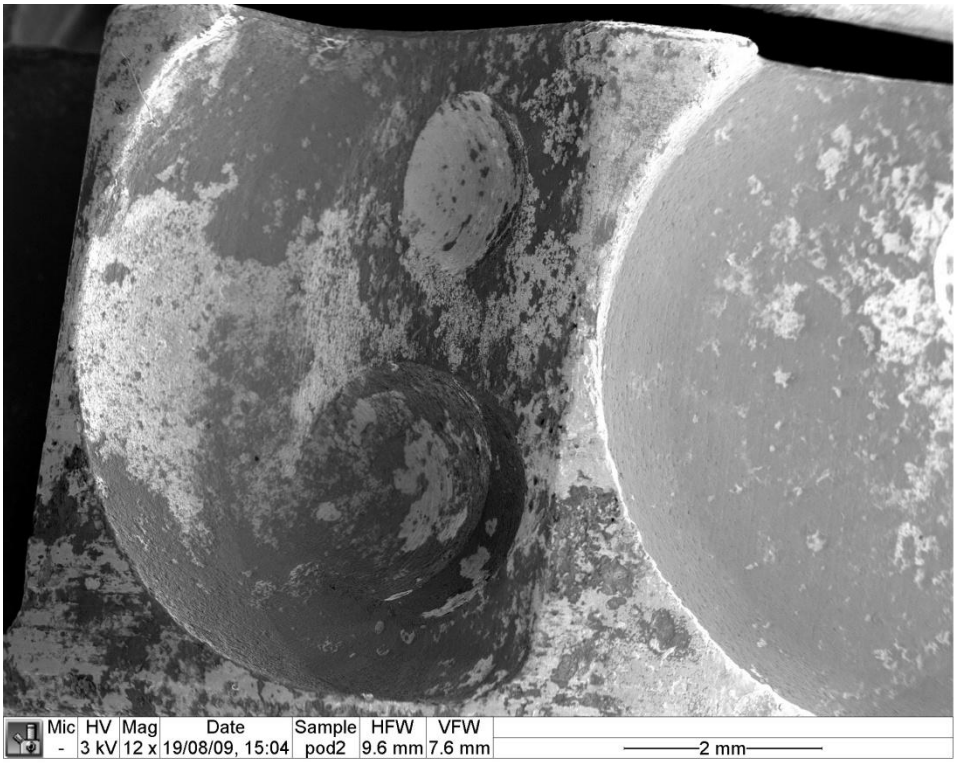
The aim of this study was to undertake a detailed surface analysis of dental HP parts before and after decontamination procedures to detect contamination on the surface. This study aimed to provide a detailed visualisation of the surface to detect contaminants invisible to routine visual analysis and to undertake a quantitative analysis of protein contamination without relying on the desorption of protein surface.

6.2 **Results**

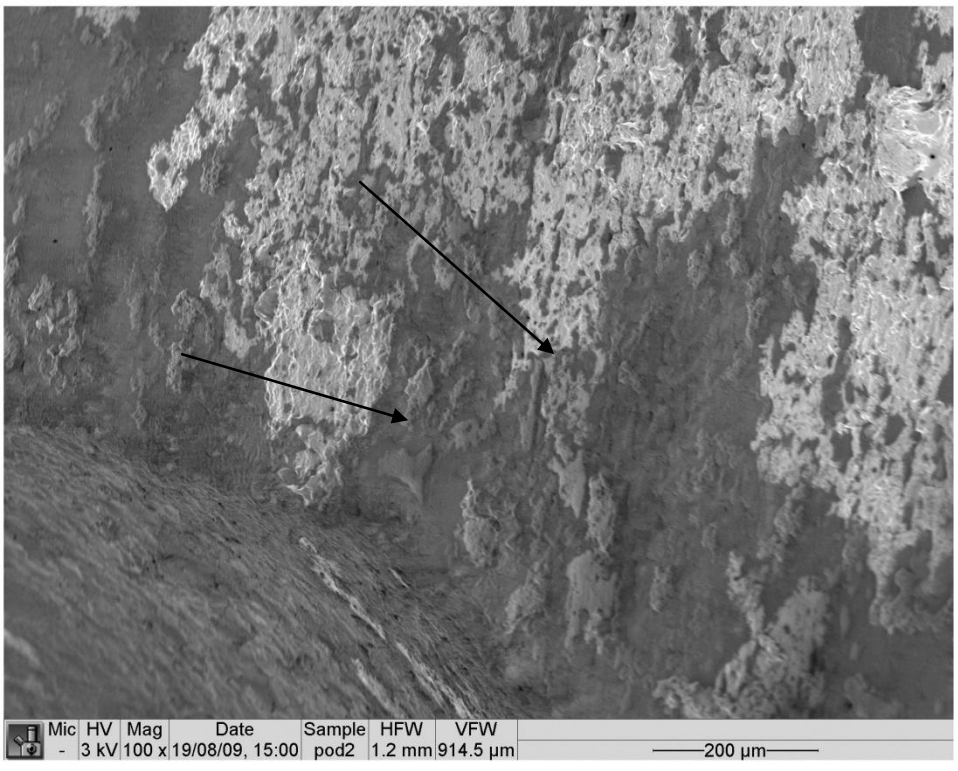
6.2.1 ***Scanning electron microscopy***

A high speed turbine was analysed for surface contamination using scanning electron microscopy without prior fixation. Additionally, a high-speed turbine and low-speed upper gear from St Albans repair facility underwent sample fixation for biological material before analysis (Section 2.4.1.1).

The SEM analysis without prior fixative showed contamination on the turbine blade (Figure 6-1 [a] [b]). A turbine from a WA- 56 high speed HP (Figure 6-2 [a]) and an upper gear of a WA – 56 low speed HP (Figure 6-3[a]) were subjected to sample fixation (Section 2.4.1.1) to allow the detection of biological material using the SEM. Contamination, including bacteria was present on the blade of the high speed turbine (Figure 6-2 [b][c]) and the ball bearing gear (Figure 6-3 [a],[b],[c]). Contamination was also visible on the low speed gear (Figure 6-4 [a], [b],[c]).



a)



b)

Figure 6-1 SEM images of a used, unprocessed high-speed turbine. The turbine was imaged without prior fixation at 120x magnification **(a)** and 1000x **(b).** The arrows indicate potential contaminants on the surface.

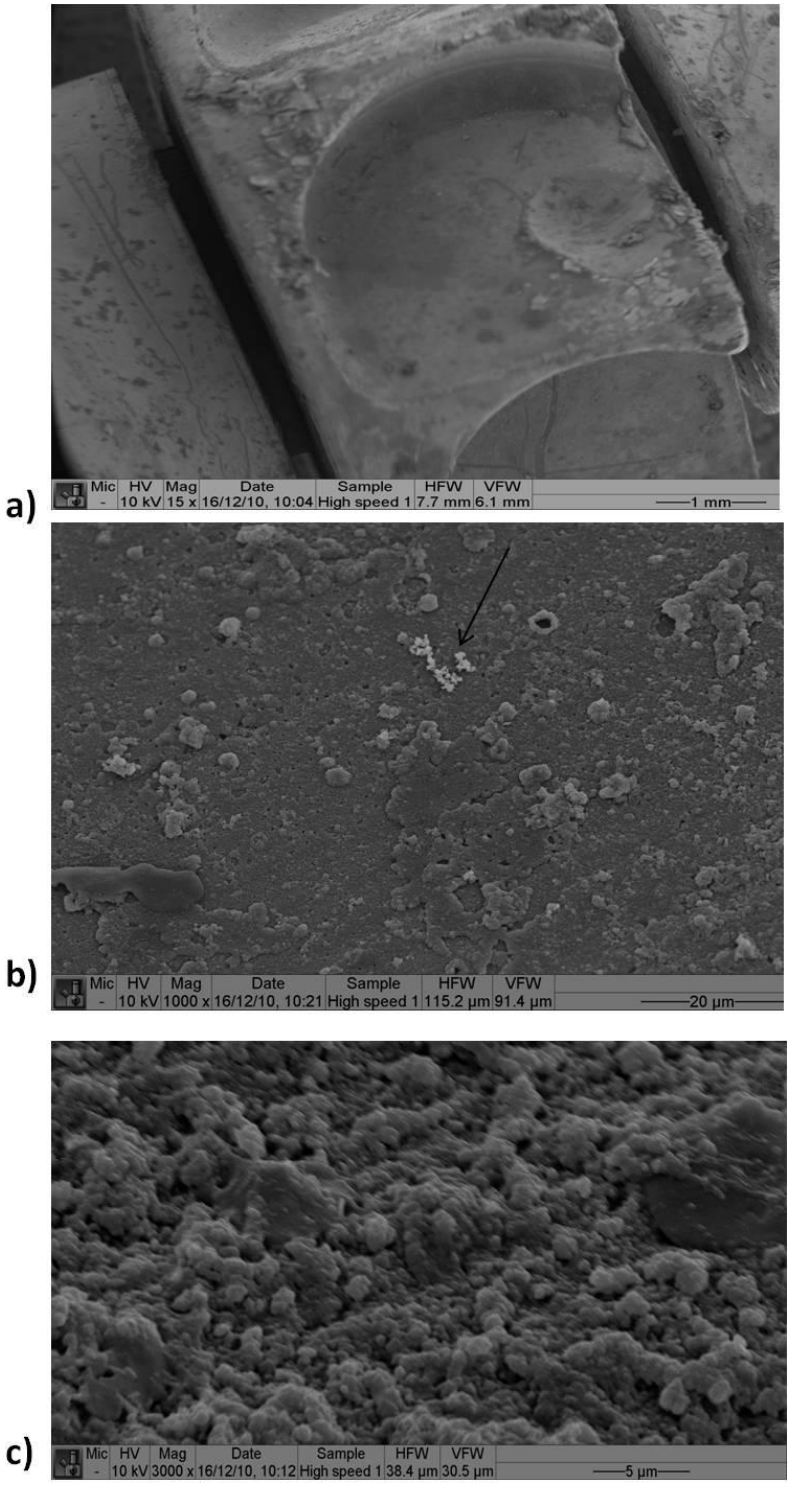


Figure 6-2 SEM imaging of TA-98 high speed turbine from a repair facility. The biological material on the turbine surface was fixed and the turbine viewed at 15x magnification for an overview **(a)** and the blade was viewed at 1000x magnification where contamination was observed on the surface **(b).** Contamination was evident along the entire surface of the turbine blade at 3000x magnification **(c).**

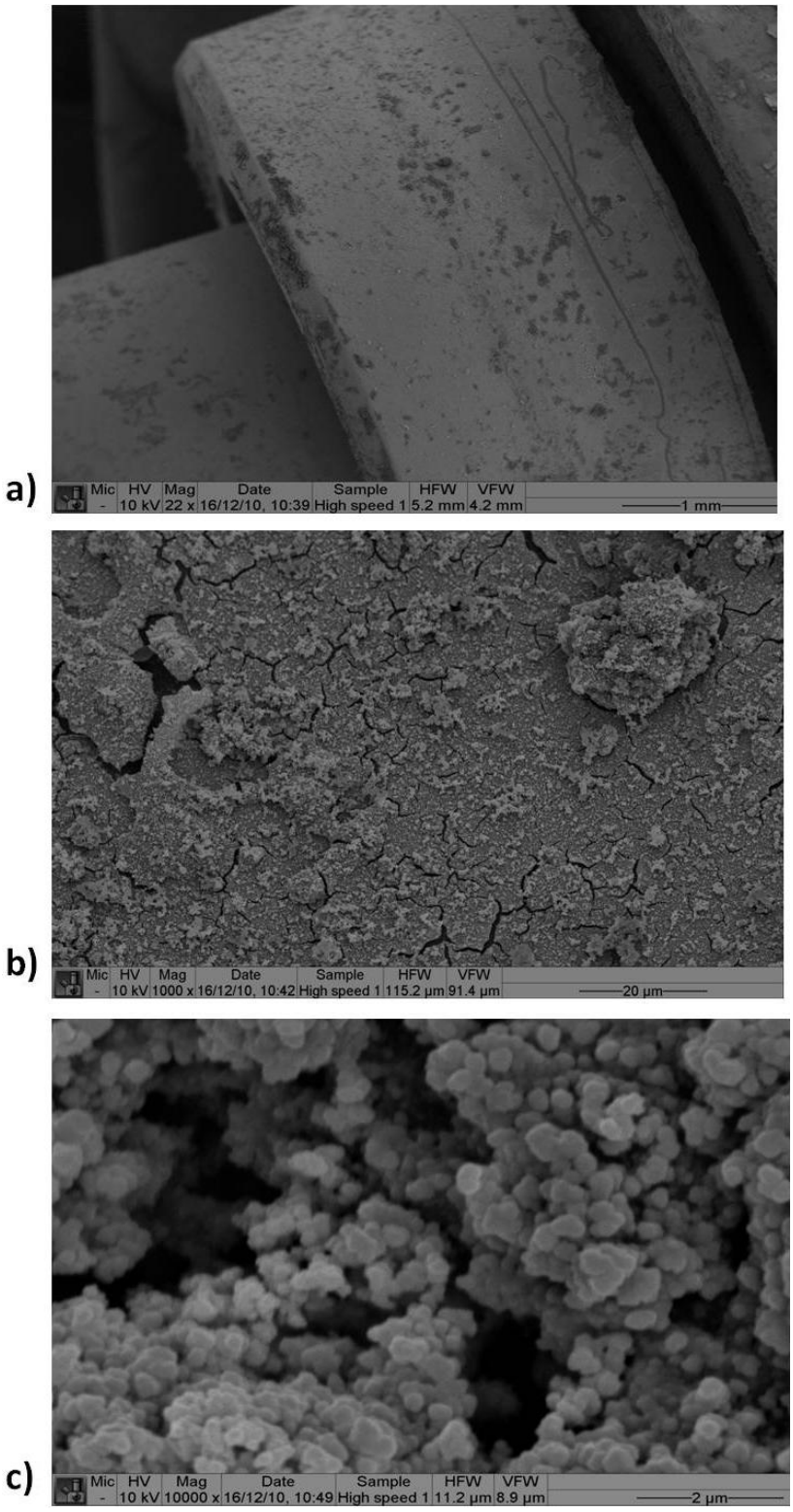


Figure 6-3 Ball bearing cage of turbine from TA-98 high-speed turbine. The ball bearing gear was visualised at 22x magnification after fixation of biological material **(a).** Contamination was visible on the surface of the ball bearing gear at 1000x magnification **(b).** Contaminants were also viewed at 10000x magnification **(c).**

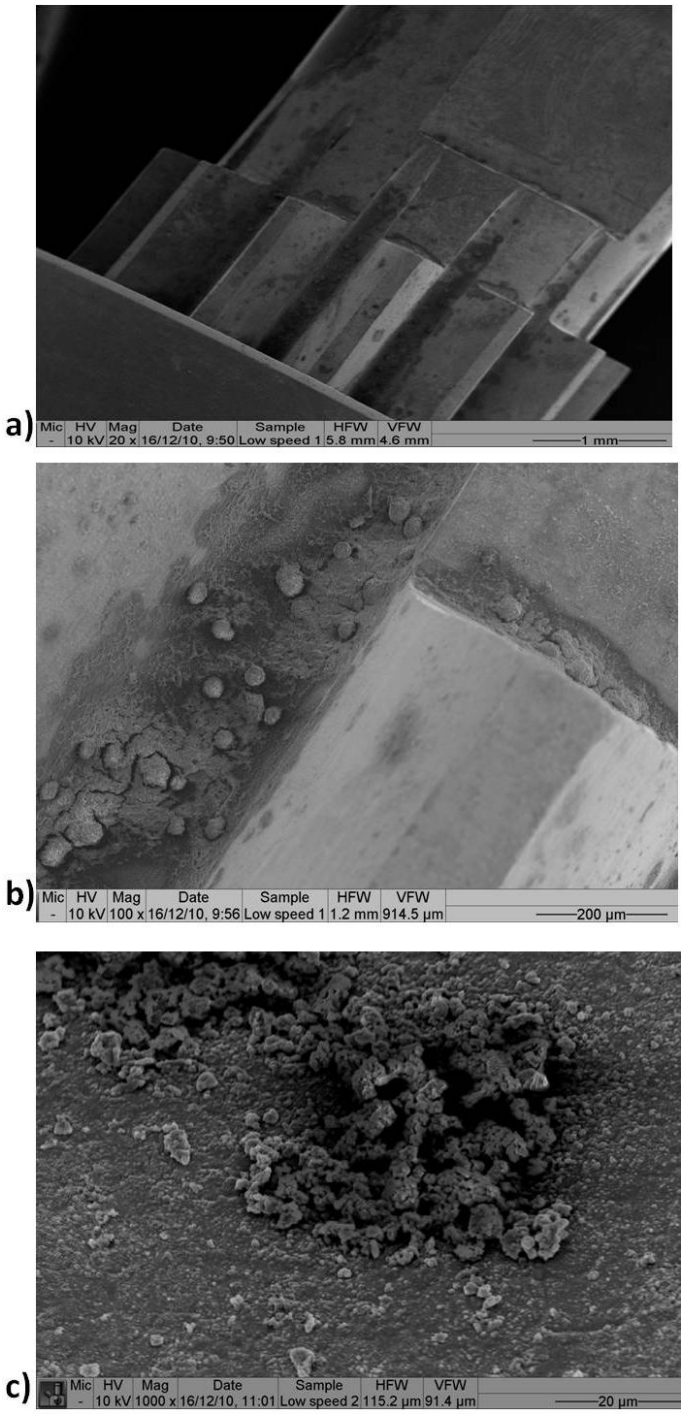


Figure 6-4 SEM images of WA- 56 low speed gear from repair facility. The biological material on the higher gear surface was fixed and the turbine viewed at 22x magnification for an overview **(a)** and the blade was viewed at 1000 x magnification where contamination was observed on the hollow parts of the gear **(b).** Contamination was also evident along the entire surface of the turbine blade at 3000x magnification **(c).**

6.2.2 *Scanning electron microscopy and energy dispersive x ray analysis*

A total of 3 used unprocessed and 3 decontaminated (Table 2.2) high speed turbines, 1 used unprocessed and 1 decontaminated high speed caps, 1 used unprocessed surgical gear and 1 decontaminated surgical gear, 1 used unprocessed surgical lever and 1 decontaminated surgical lever, and 1 repair facility high gear and 1 used, unprocessed high gear were analysed. Parts from unused HP's that had been through a decontamination process were sampled as negative controls.

For the high speed HP's, contamination was detected on the high speed turbines (Figure 6-5 [a] [b]), and the used, unprocessed and decontaminated high speed caps (Figure 6-5 [c],[d]). For surgical HP's, contamination was detected on gears (Figure 6-6 [a],[b],[c],[d]), and used, unprocessed levers (Figure 6-6 [c],[d]). For low speed HPs, contamination was detected on used, unprocessed higher gears (Figure 6-7 [a],[b]) and used, unprocessed and decontaminated lower gears (Figure 6-7 [c],[d]).

Contamination was subdivided into organic contamination indicated by dominant carbon and oxygen peaks (Figure 6-8 [a]), calcium deposits indicated by dominant calcium, oxygen and carbon peaks (Figure 6-8 [b]), sulphur organic contamination indicated by dominant carbon, oxygen and sulphur peaks (Figure 6-8 [c]) and lubricating oil trapped contaminants indicated by a carbon based peak with visible contaminants in contact (Figure 6-8 [d]). Analysis of non-contaminated surfaces adjacent to contamination revealed metallic elements with different composition to each contaminant (Figure 6-9). All contaminant groups were detected on high speed turbines before and after decontamination (Figure 6-10 [a],[b],[c],[d], Figure 6-11[a],[b],[c],[d])); surgical gears before and after decontamination (Figure 6-14 [a],[b],[c],[d], Figure 6-15 [a],[b],[c],[d]); surgical levers before decontamination (Figure 6-16 [a],[b],[c],[d]); low speed upper gears before decontamination (Figure 6-18 [a],[b],[c],[d]) and low speed upper gears before and after decontamination (Figure 6-19 [a],[b],[c],[d],Figure 6-20 [a],[b],[c],[d]).). Only organic contamination was found on high speed caps before and after decontamination (Figure 6-12 [a],[b], Figure 6-13[a],[b]) and

only organic contamination, lubricating oil trapped contaminants and sulphur based contaminants were found on decontaminated surgical levers (Figure 6-17 [a],[b],[c]).

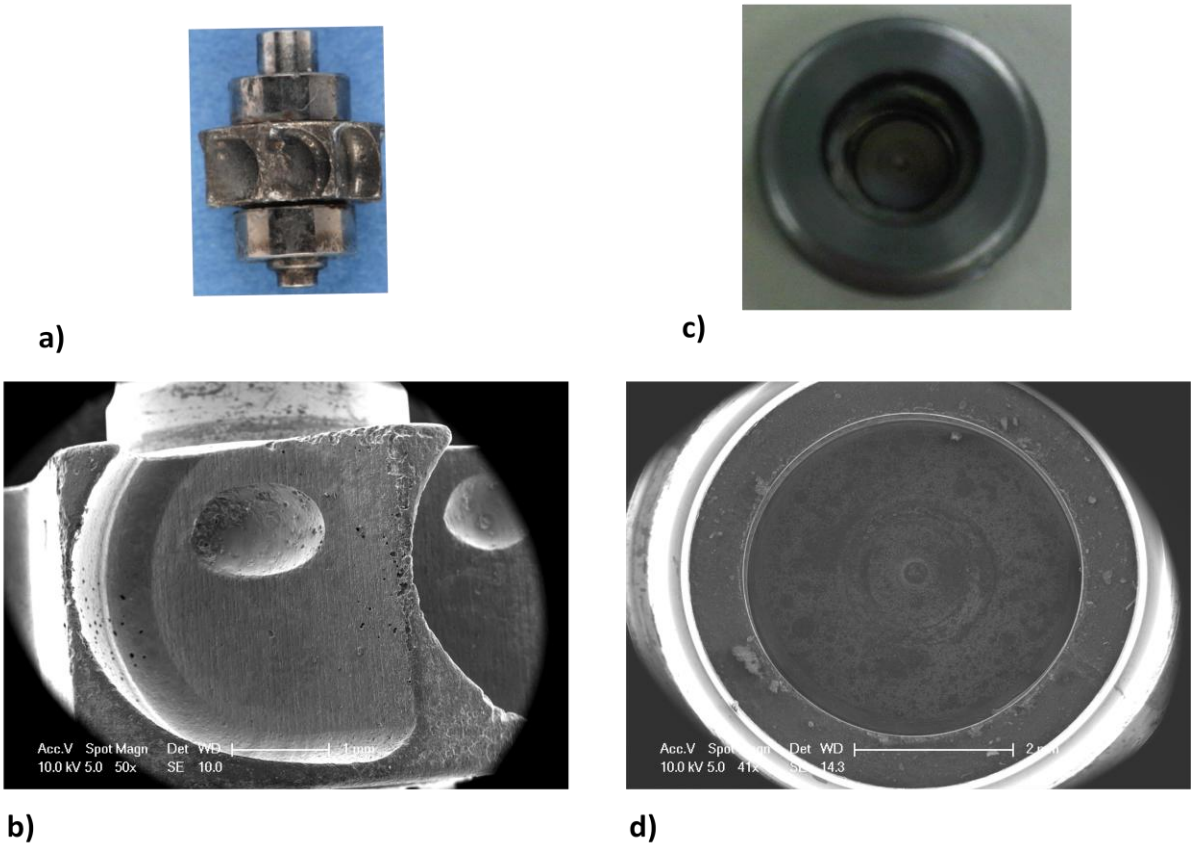


Figure 6-5 High speed HP parts sampled using SEM and EDX analysis. Both high speed turbines **(a, b)** and caps **(c, d)** were sampled using SEM and EDX analysis. A microscopic view of the turbine is shown in **(c)** at 50x magnification and a microscopic view of the cap is shown in **(d)** at 41x magnification.

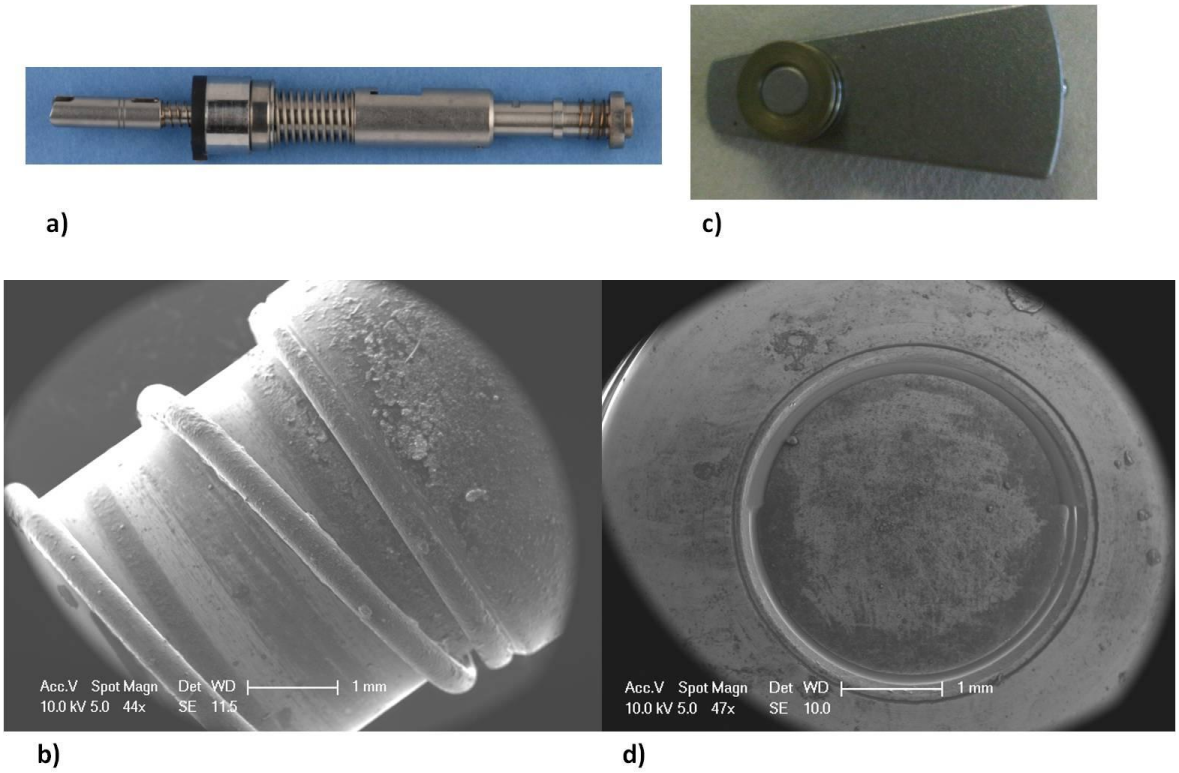


Figure 6-6 Surgical HP parts sampled using SEM and EDX analysis. Both surgical HP gears **(a, b)** and the levers **(c, d)** were sampled using SEM and EDX analysis. A microscopic view of the spring of the gear is shown in **(c)** at 44x magnification and a microscopic view of the internal part of the lever is shown in **(d)** at 47x magnification.

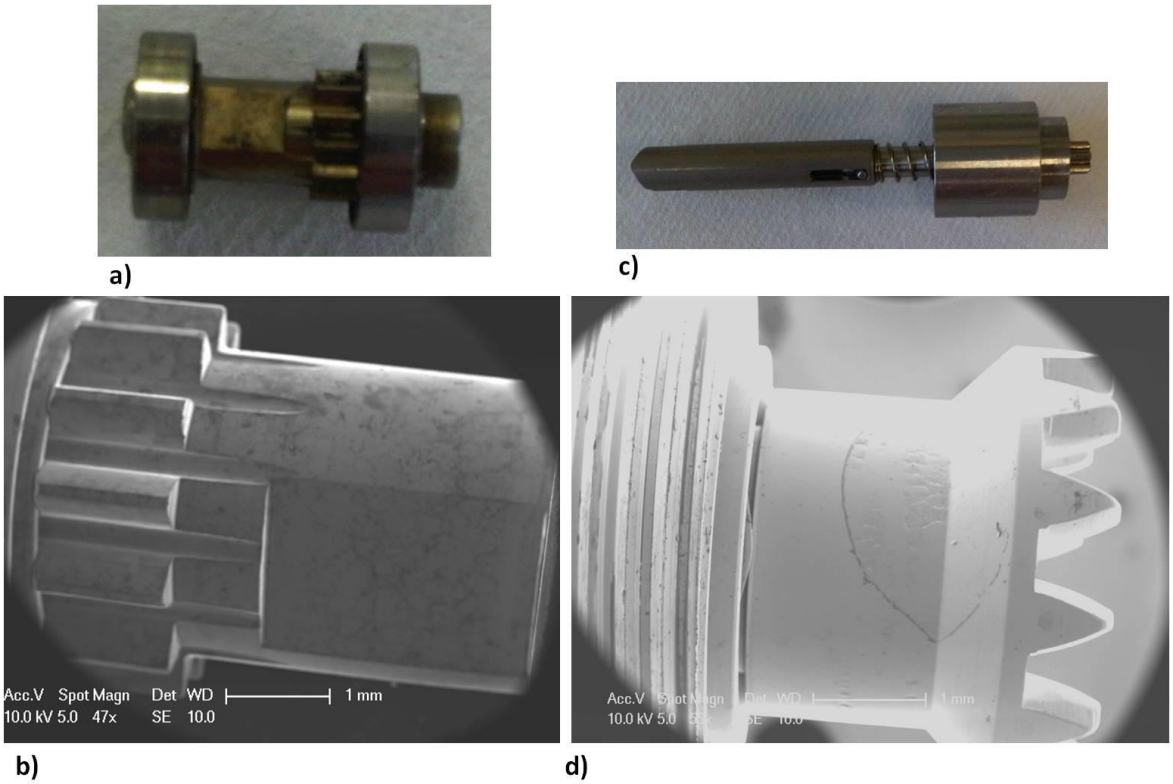


Figure 6-7 Low speed HP parts sampled using SEM and EDX analysis. Both low speed higher gears **(a, b)** and lower gears **(c, d)** were sampled using SEM and EDX analysis. A microscopic view of the gear is shown in **(c)** at 50x magnification and a microscopic view of the end of the lower gear is shown in **(d)** at 55x magnification.

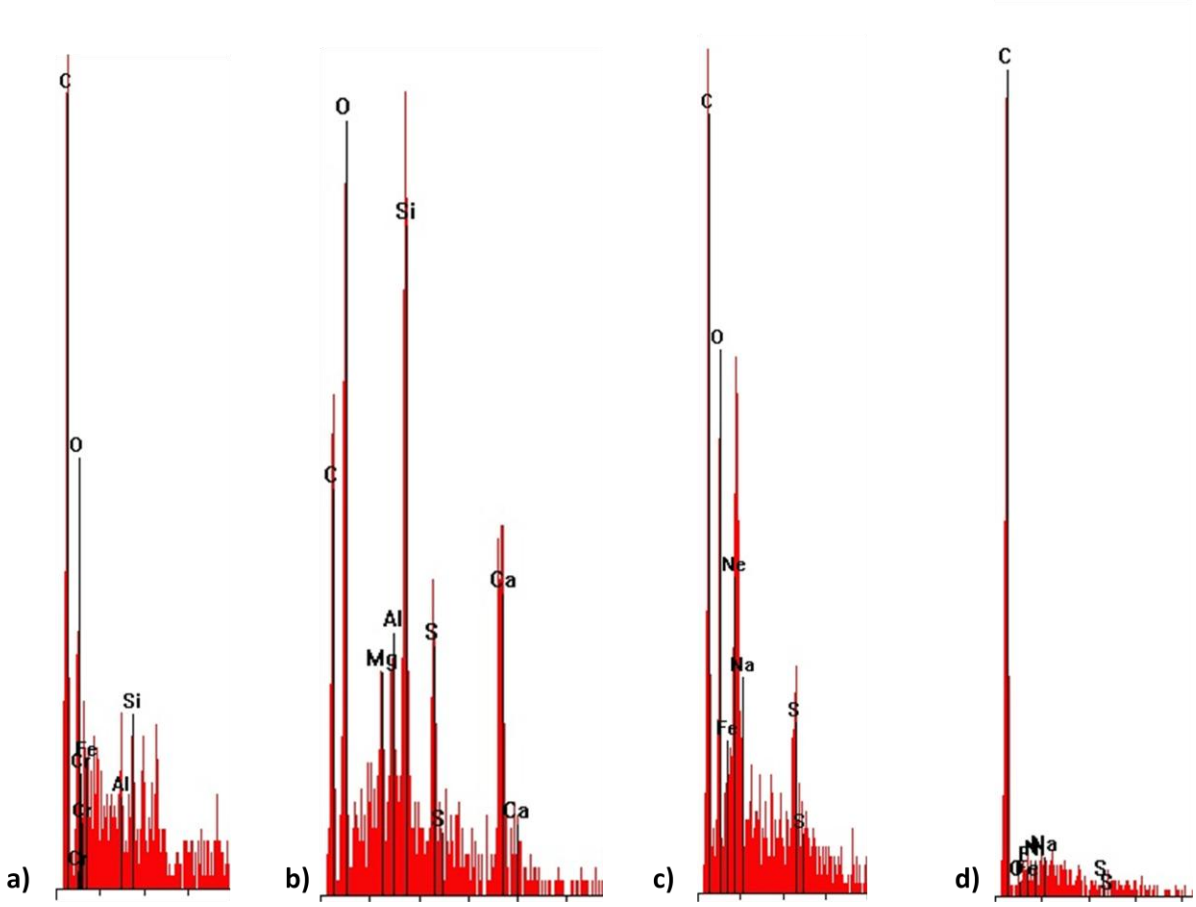


Figure 6-8 Typical elemental analysis of HP surface contaminants by EDX analysis. All contaminants were broadly placed into 4 categories, organic contamination **(a)** indicated by the carbon and oxygen peaks, calcium based contamination **(b)** indicated by a calcium peak, sulphur based contamination **(c)** indicated by carbon, oxygen, and sulphur peaks and carbon based contamination **(d)** indicated by a carbon peak. Each contaminant was found on all surfaces scanned.

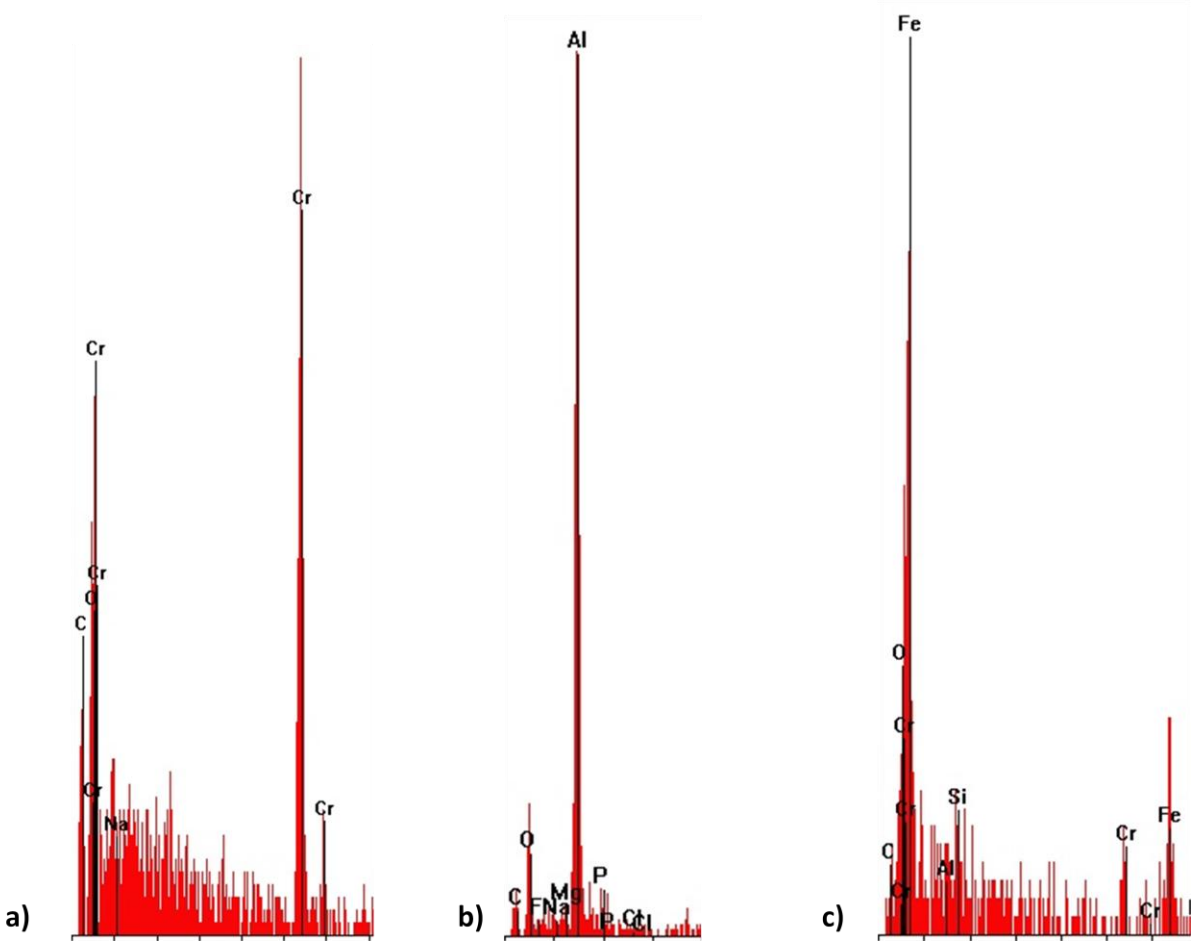


Figure 6-9 Typical elemental analysis of HP metallic surfaces by EDX analysis. Surfaces in the vicinity of contamination were analysed as controls. Metallic elements including chromium **(a)** found on the lower gear, aluminium **(b)** found on the high speed turbine, and iron **(c)** found on the surgical gear and high speed caps.

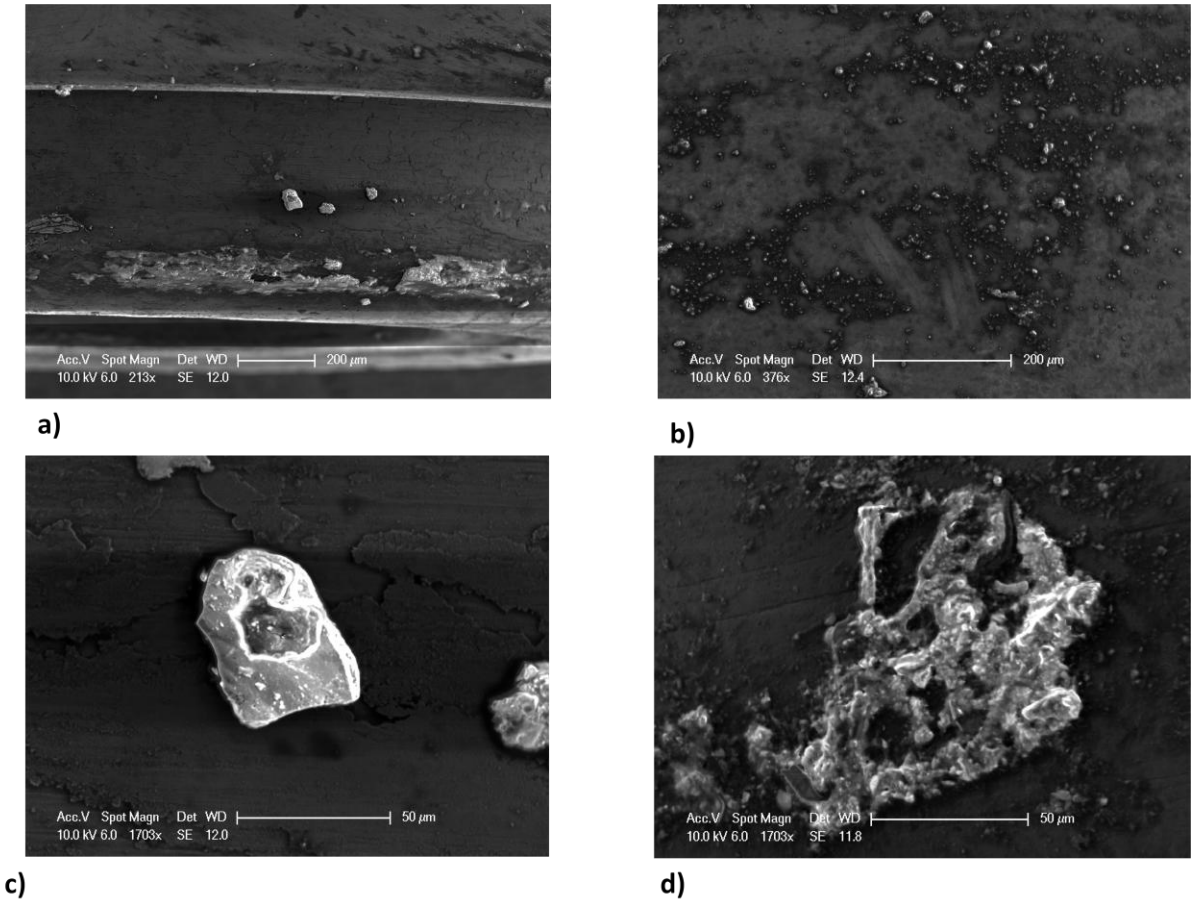


Figure 6-10 Examples of contaminants found on used, unprocessed high speed turbines. All contaminants previously described were found in the high speed turbine ball bearing cage before decontamination. Organic contamination is shown in **(a)** at 213x magnification, lubricating oil trapped contamination is shown in **(b)** at 376 x contamination**,** a calcium contaminant shown in **(c)** at 1703 x magnification and sulphur based contamination is shown in **(d)** at 1700x magnification.

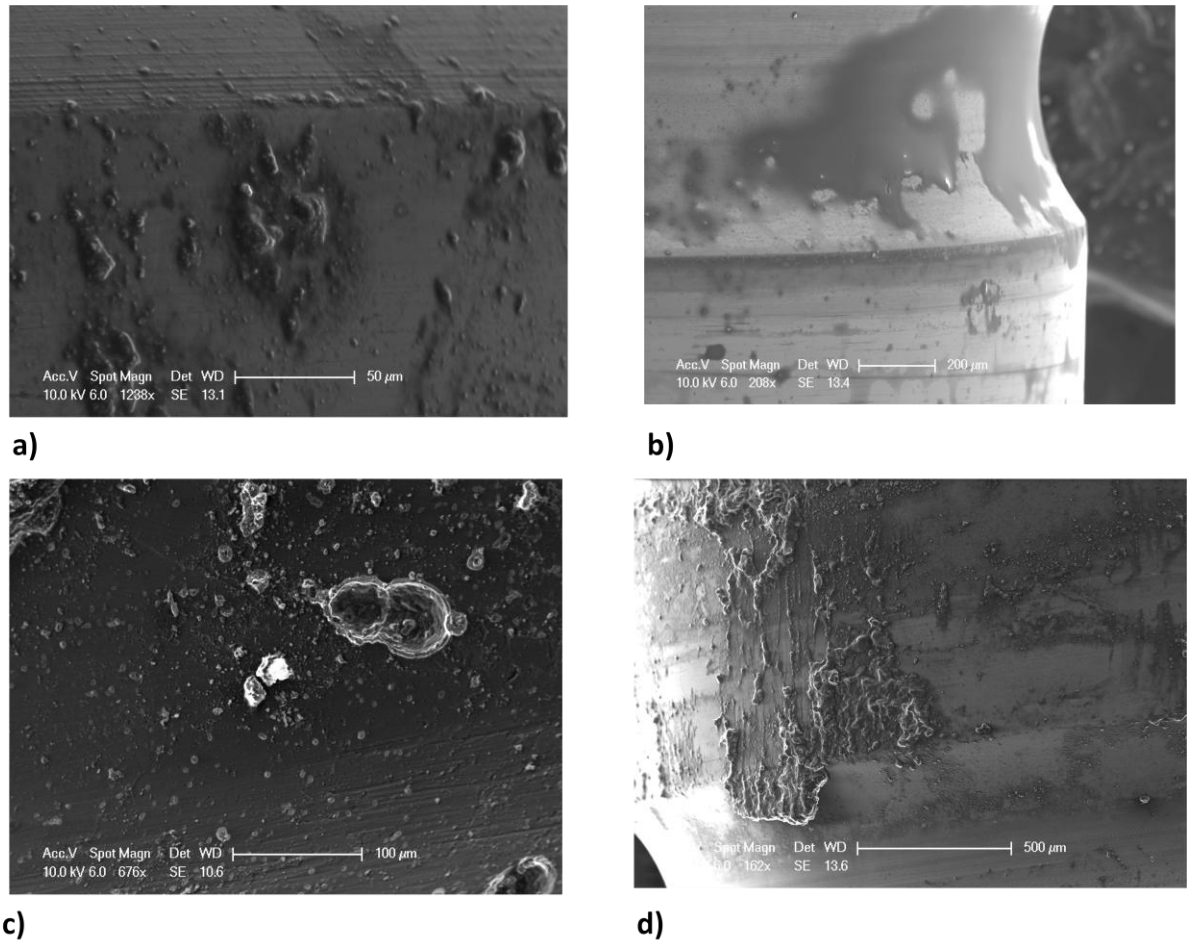


Figure 6-11 Examples of contaminants found on decontaminated high speed turbines. All contaminants previously described were found in the high speed turbine ball bearing cage before decontamination. Organic contamination is shown in **(a)** at 1238x magnification, lubricating oil trapped contamination is shown in **(b)** at 208 x contamination**,** a calcium contaminant shown in **(c)** at 676 x magnification and sulphur based contamination is shown in **(d)** at 162x magnification.

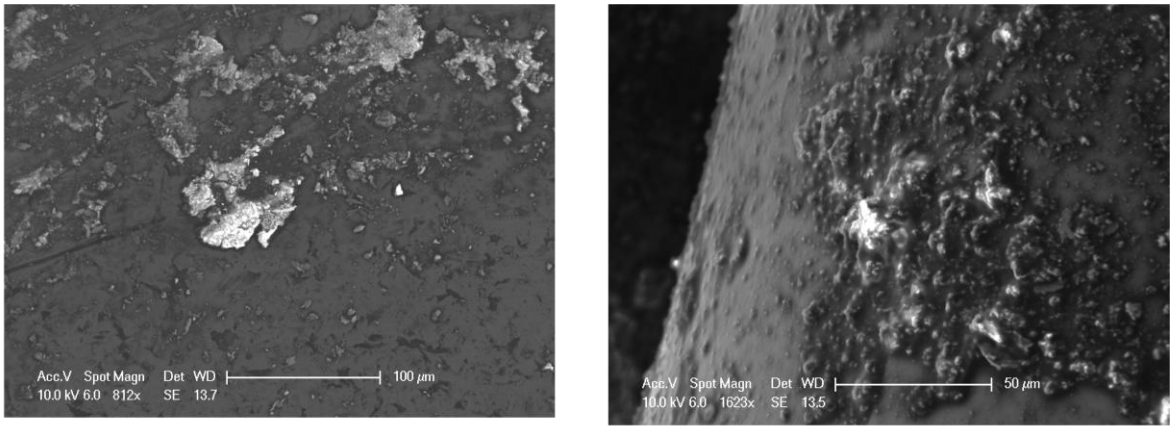


Figure 6-12 Examples of contaminants found on used, unprocessed high speed caps. Organic contaminants previously described were found on the high speed cap before decontamination. Organic contamination is shown in **(a)** at 812 x magnification and in **(b)** at 1623 x magnification.

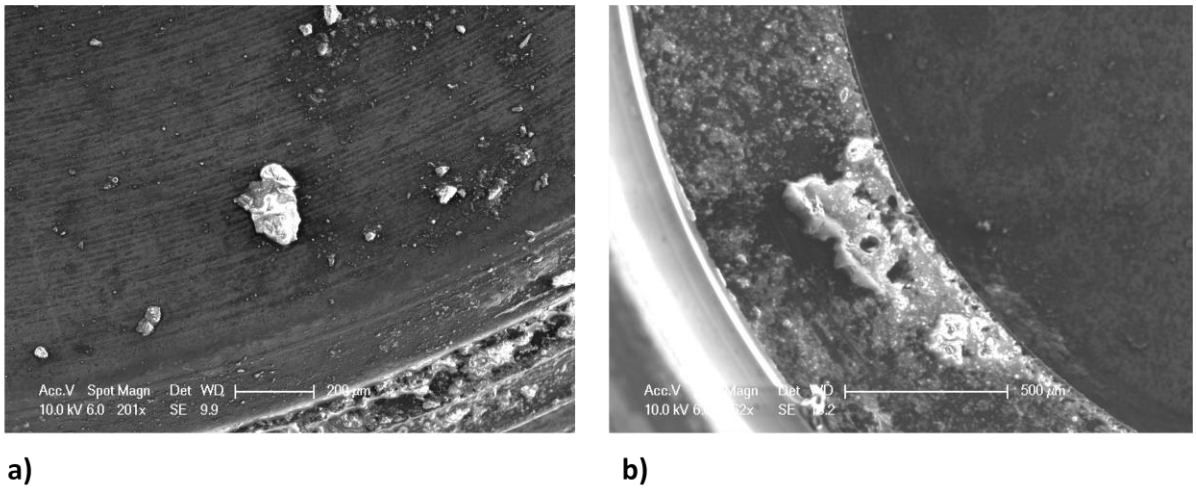


Figure 6-13 Examples of contaminants found on decontaminated high speed caps. Organic contaminants previously described were found on the high speed cap before decontamination. Organic contamination is shown in **(a)** at 201 x magnification and in **(b)** at 162 x magnification.

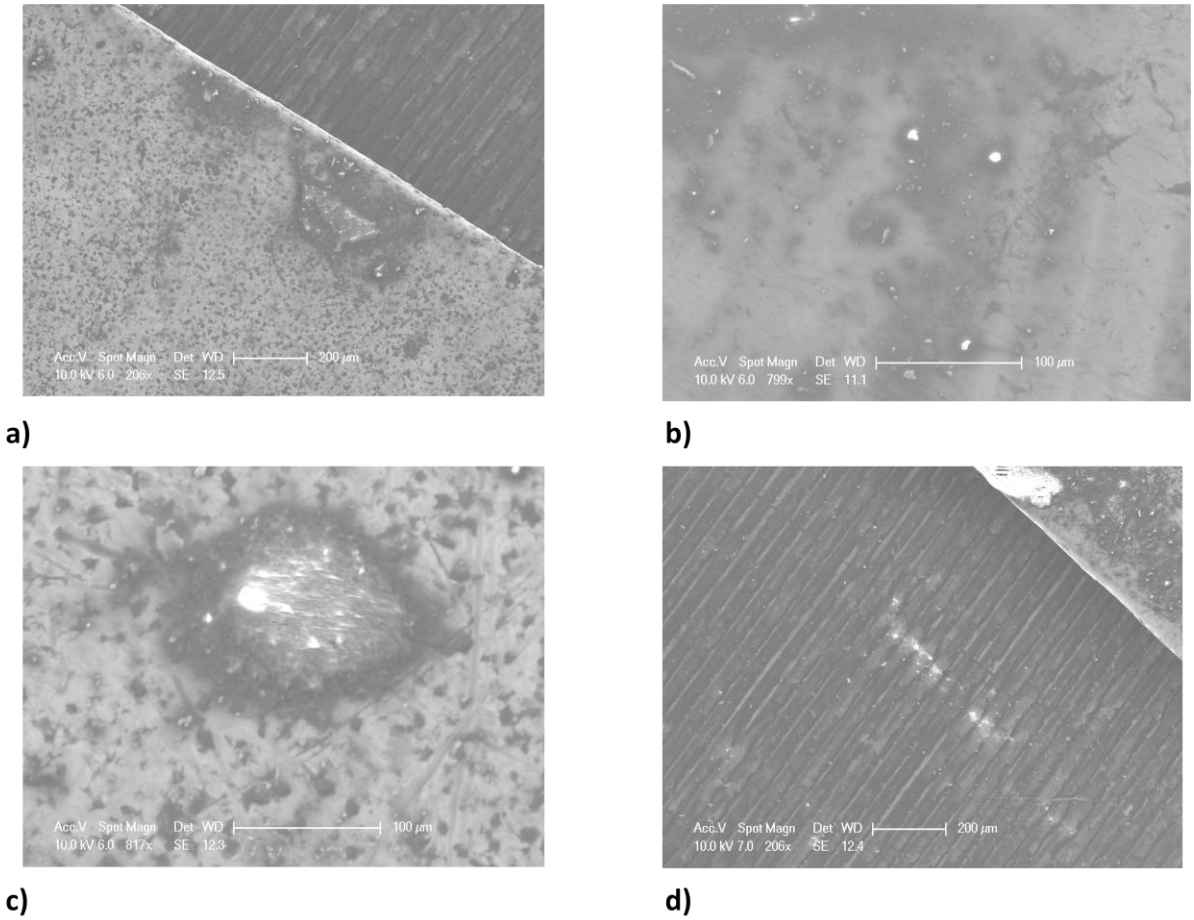


Figure 6-14 Examples of contaminants found on used, unprocessed surgical gears. All contaminants previously described were found in the gear before decontamination. Organic contamination is shown in **(a)** at 206 x magnification, lubricating oil trapped contamination is shown in **(b)** at 790 x contamination**,** a calcium contaminant shown in **(c)** at 817 x magnification and sulphur based contamination is shown in **(d)** at 206 x magnification.

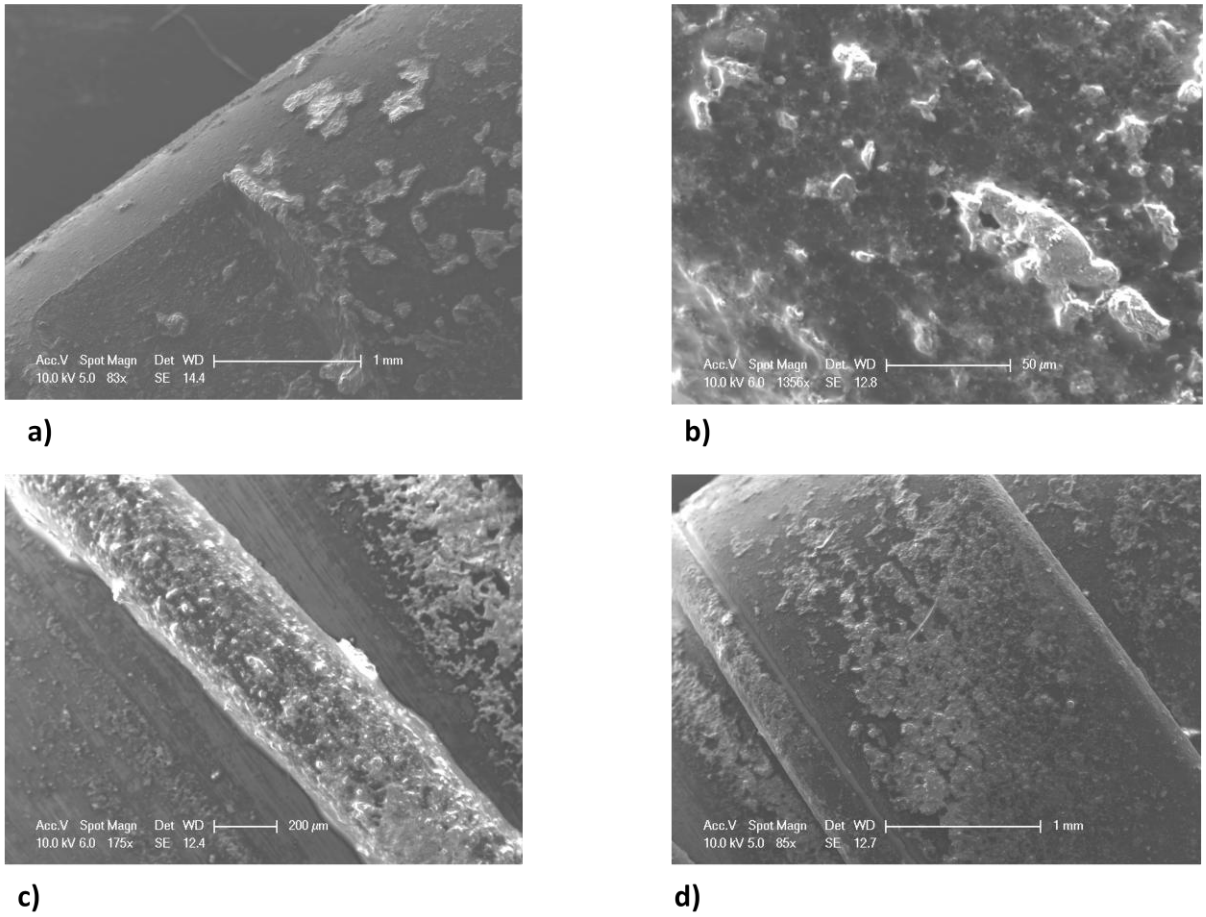


Figure 6-15 Examples of contaminants found on decontaminated surgical gears. All contaminants previously described were found on the spring before decontamination. Organic contamination is shown in **(a)** at 83 x magnification, lubricating oil trapped contamination is shown in **(b)** at 1356 x contamination**,** a calcium contaminant shown in **(c)** at 175 x magnification and sulphur based contamination is shown in **(d)** at 85 x magnification.

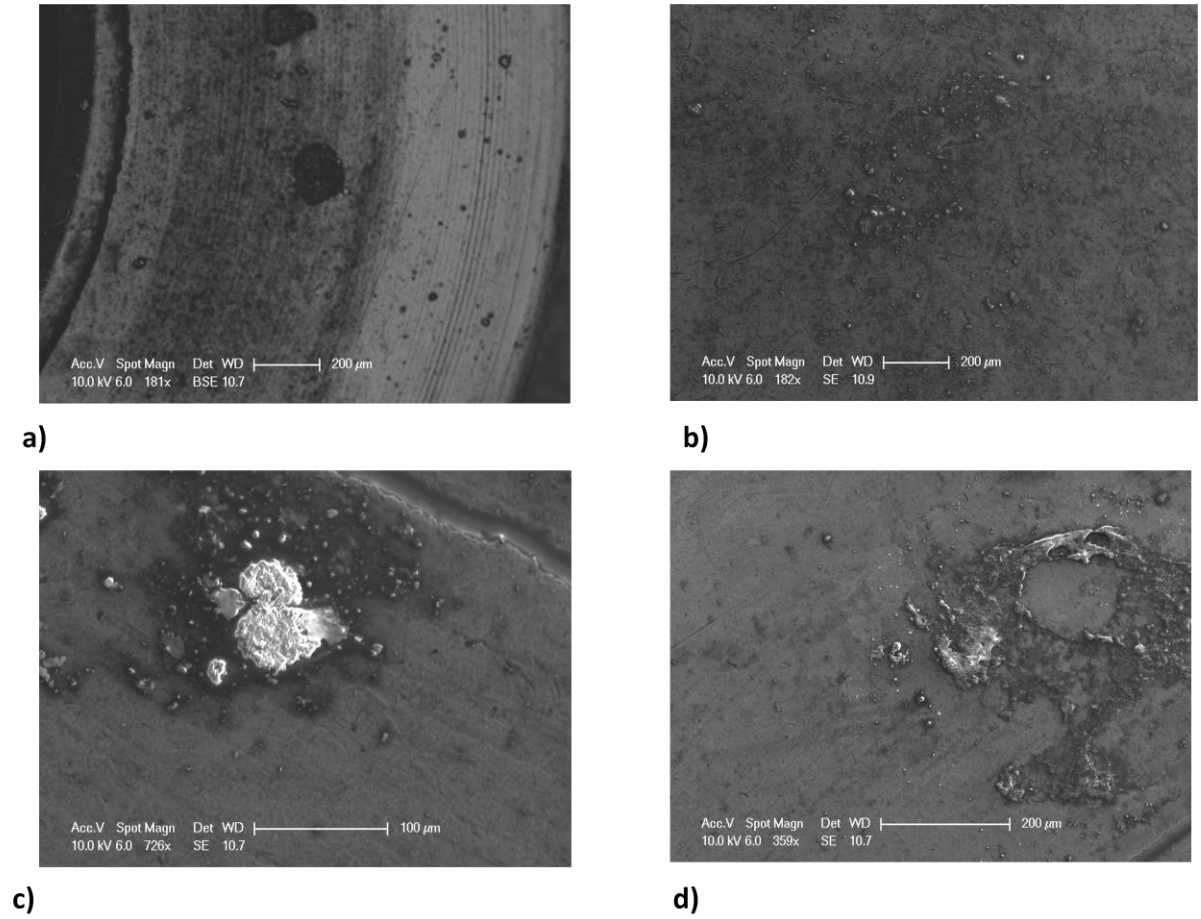


Figure 6-16 Examples of contaminants found on used, unprocessed surgical levers. All contaminants previously described were found on the lever in contact with the gear before decontamination. Organic contamination is shown in **(a)** at 181 x magnification, lubricating oil trapped contamination is shown in **(b)** at 726 x contamination**,** a calcium contaminant shown in **(c)** at 175 x magnification and sulphur based contamination is shown in **(d)** at 359 x magnification.

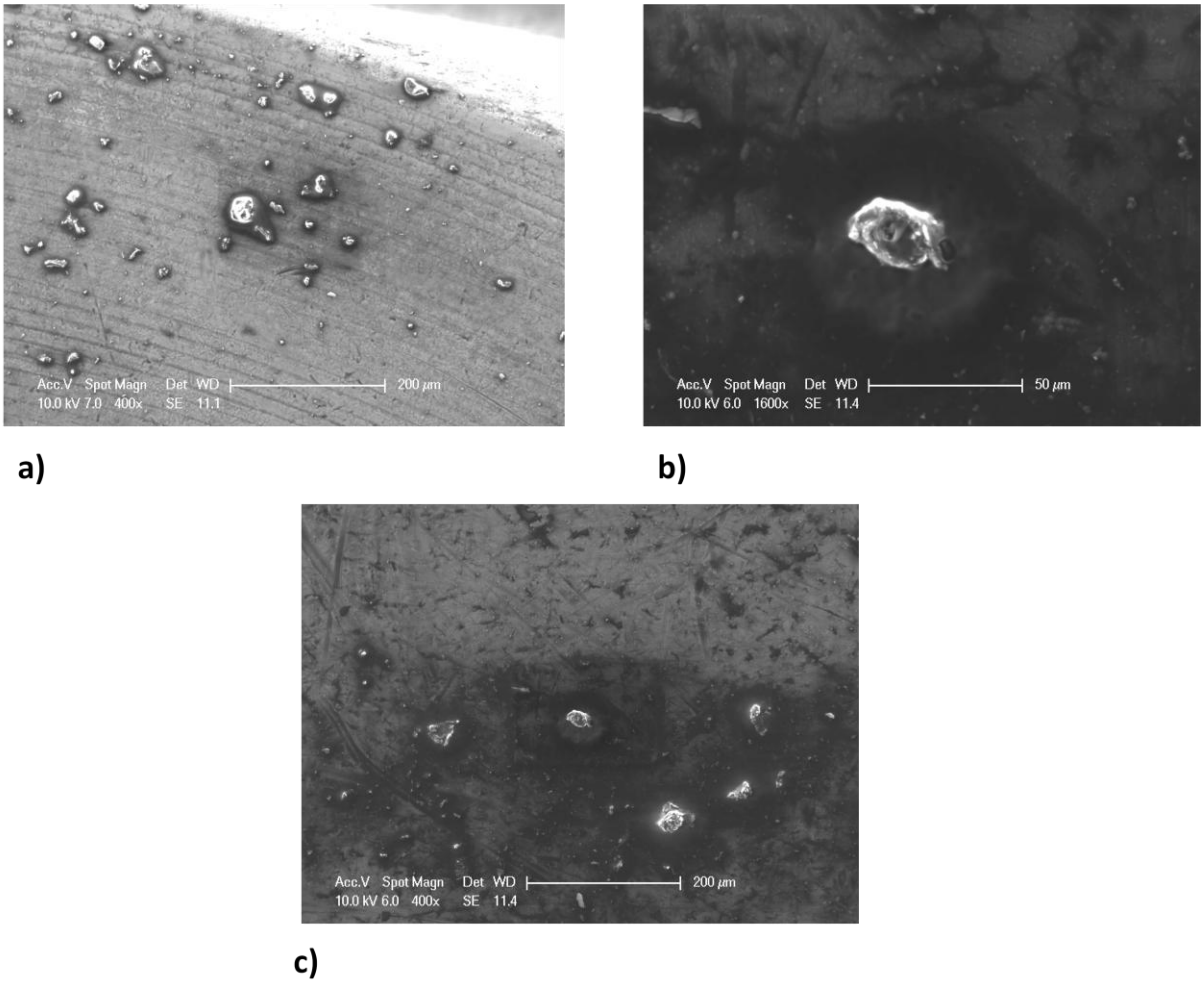


Figure 6-17 Examples of contaminants found on decontaminated surgical levers. Contaminants previously described were found on the lever in contact with the gear before decontamination. Organic contamination is shown in **(a)** at 400 x magnification, sulphur based contamination is shown in **(b)** at 1600 x magnification and lubricating oil trapped contamination is shown in **(c)** at 400x magnification. No calcium based contamination was observed.

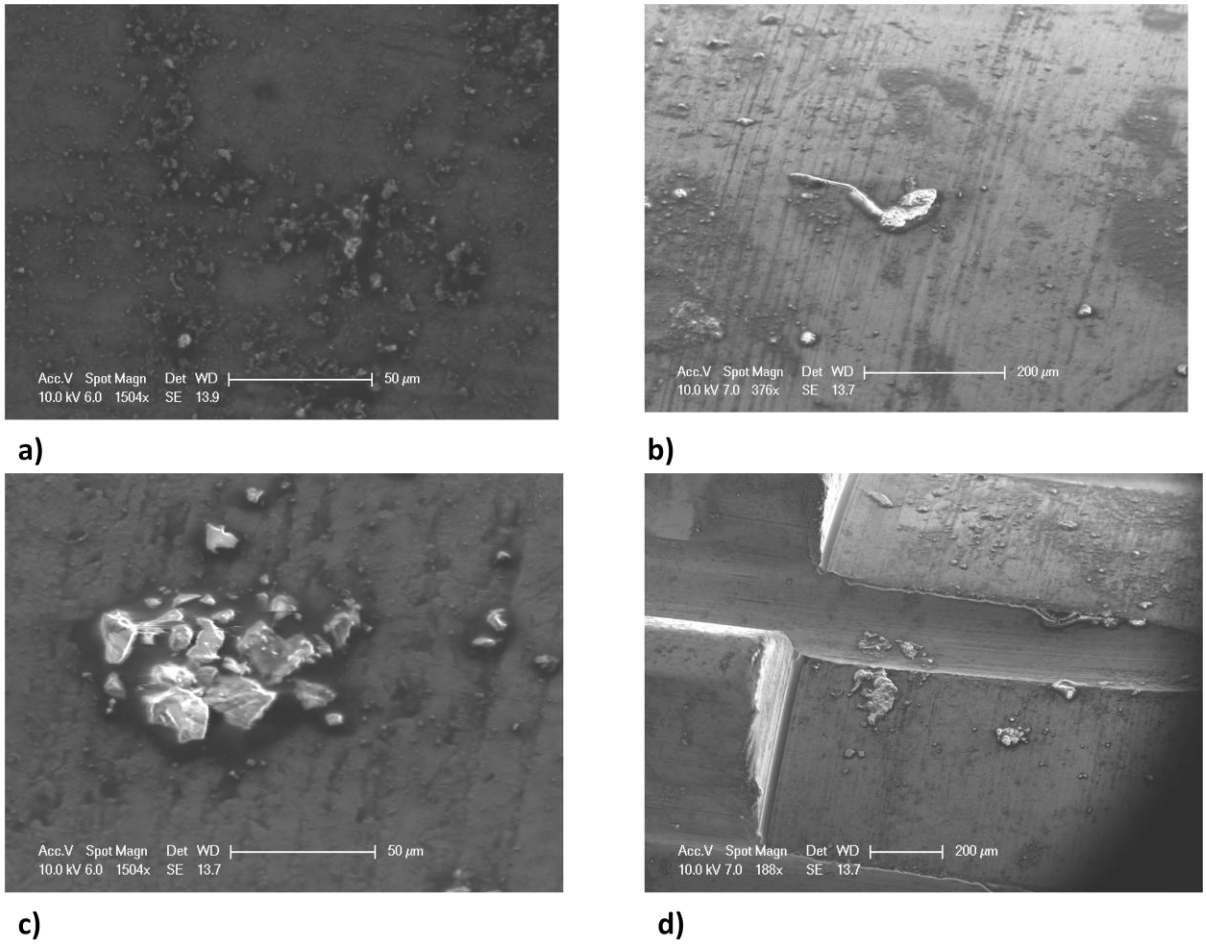


Figure 6-18 Examples of contaminants found on used, unprocessed low speed upper gears. All contaminants previously described were found in the high speed turbine ball bearing gear before decontamination. Organic contamination is shown in **(a)** at 1504 x magnification, lubricating oil trapped contamination is shown in **(b)** at 376 x contamination**,** a calcium contaminant shown in **(c)** at 1504 x magnification and sulphur based contamination is shown in **(d)** at 188 x magnification.

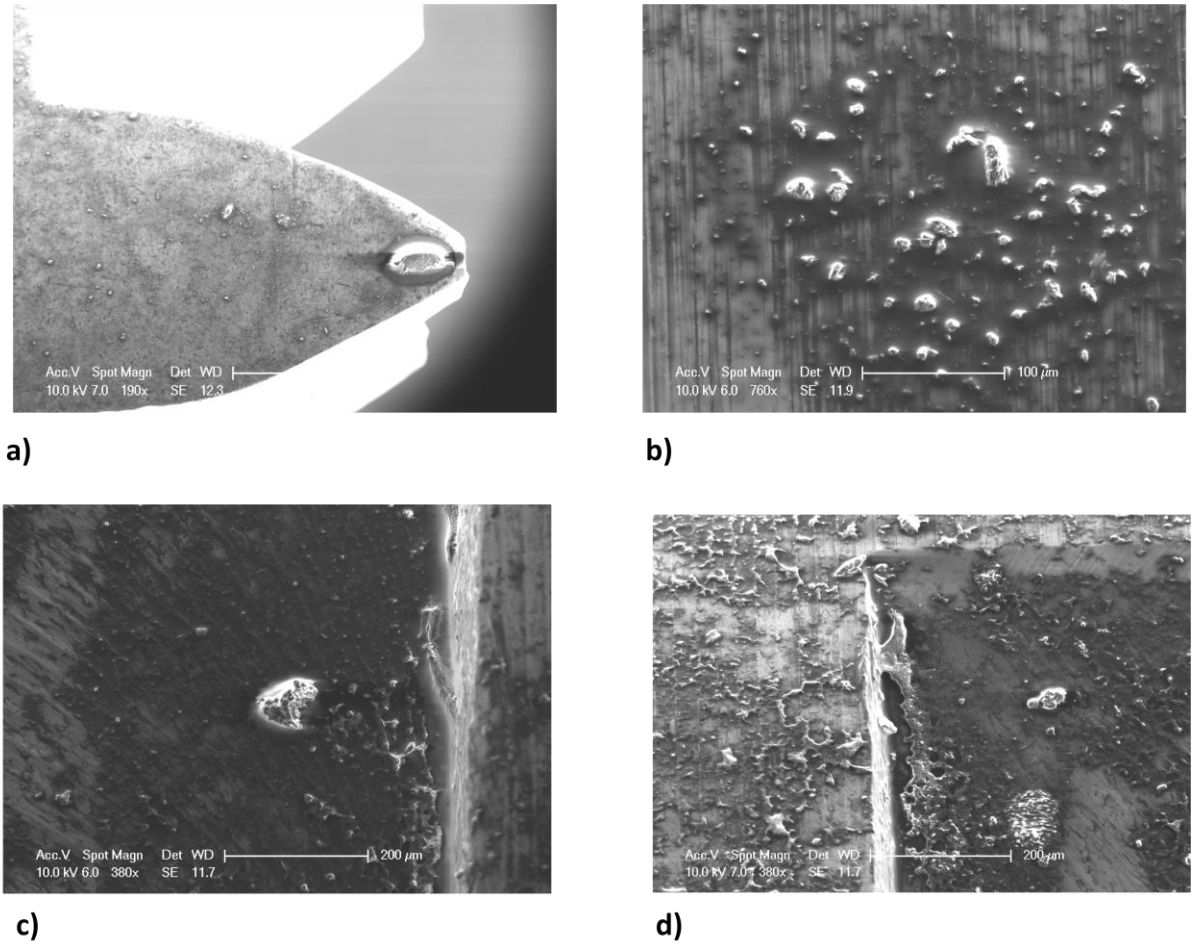


Figure 6-19 Examples of contaminants found on used, unprocessed low speed lower gears. All contaminants previously described were found in the low speed lower gear before decontamination. Organic contamination is shown in **(a)** at 190 x magnification, lubricating oil trapped contamination is shown in **(b)** at 760 x contamination**,** a calcium contaminant shown in **(c)** at 380 x magnification and sulphur based contamination is shown in **(d)** at 380 x magnification.

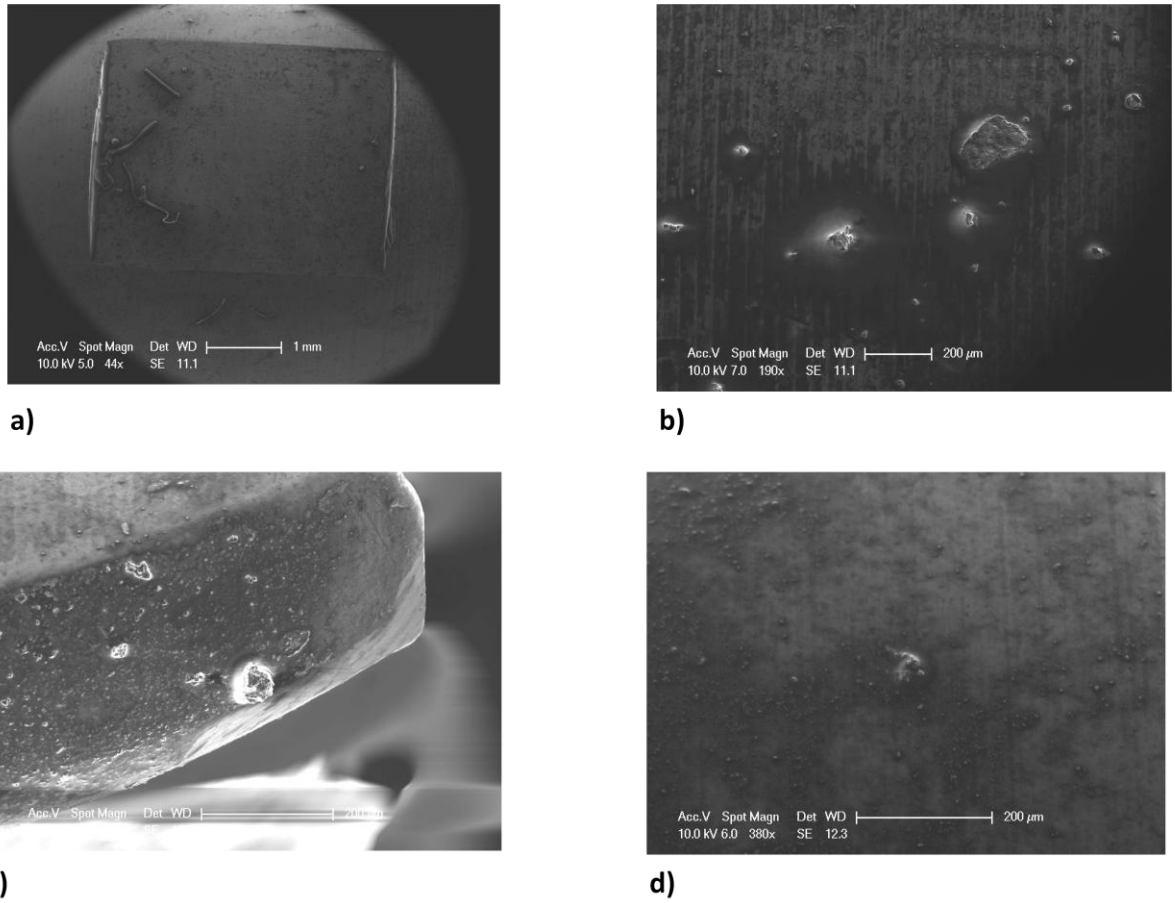


Figure 6-20 Examples of contaminants found on decontaminated low speed lower gears. All contaminants previously described were found on the low speed lower gear after decontamination. Organic contamination is shown in **(a)** at 44 x magnification, lubricating oil trapped contamination is shown in **(b)** at 190 x contamination**,** a calcium contaminant shown in **(c)** at 190 x magnification and sulphur based contamination is shown in **(d)** at 380 x magnification.

6.2.3 *Epifluorescent scanning analysis*

For quantitiative analysis of the EFSCAN samples, a 10 µl sample of BSA at concentrations 2, 1, 0.75, 0.5 mg of BSA were added to prepared SSS's (Section 2.1). The SSS's were dried at ambient room temperature overnight before sampling using EFSCAN analysis (Section 2.4.1.3). The sum of the fluorescent peaks was added together to get the value associated with that protein concentration for comparison to unknown samples.

For high speed turbines and caps, a total of 3 used unprocessed, 3 decontaminated at the GDH and 3 from the repair facility were analysed. Decontaminated and unused parts were sampled for negative controls. Additionally, a total of 3 used, unprocessed and 3 decontaminated low speed spray channels and higher gears were sampled.

An increase in BSA concentration results in a linear increase in fluorescent units (Figure 6-21, Figure 6-22). Protein was detected on high speed HP turbines (Figure 6-24 [a]) before and after decontamination () (Figure 6-24 [c],[d], Table 6-1,), high seed HP caps(Figure 6-25 [a]) before and after decontamination (Figure 6-25 [c], [d], Table 6-1)and low speed HP spray channels (Figure 6-23 [b]) before and after decontamination (Figure 6-23 [c], [d],Table 6-1). For all parts sampled, no significant reduction in protein was observed for all instruments after decontamination (Table 6-1). EFSCAN analysis of unused surfaces did not detect protein significantly greater than the negative control (Figure 6-24 [b], Figure 6-25[b], Figure 6-23[b]).

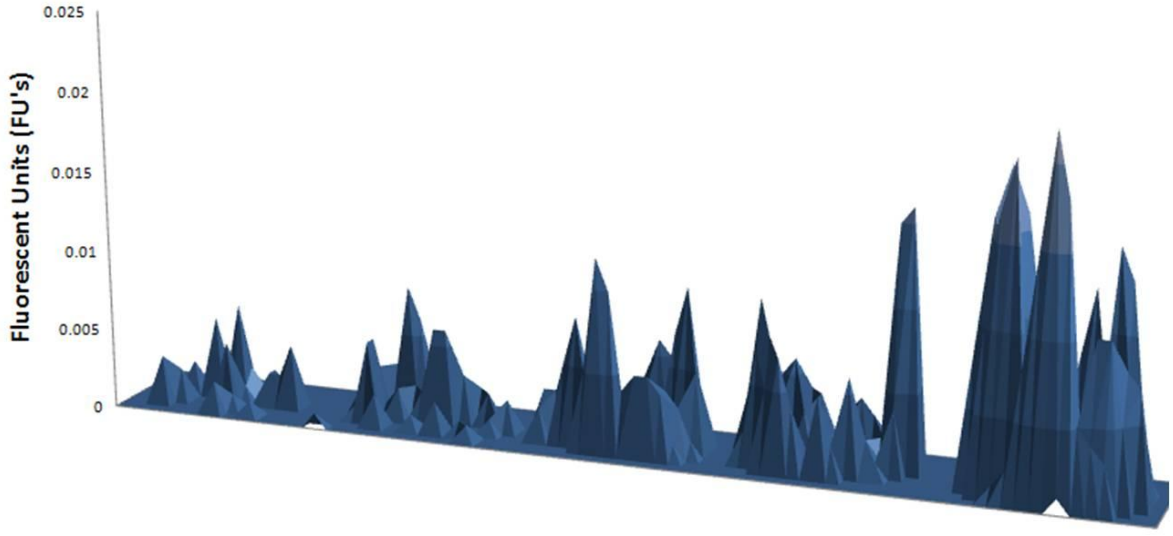


Figure 6-21 EFSCAN analysis of BSA standards on stainless steel discs. BSA of differing concentrations was applied to 316 stainless steel disc and analysed. Each peak represents protein contamination on the surface. The data is representative of 6 discs of each concentration.

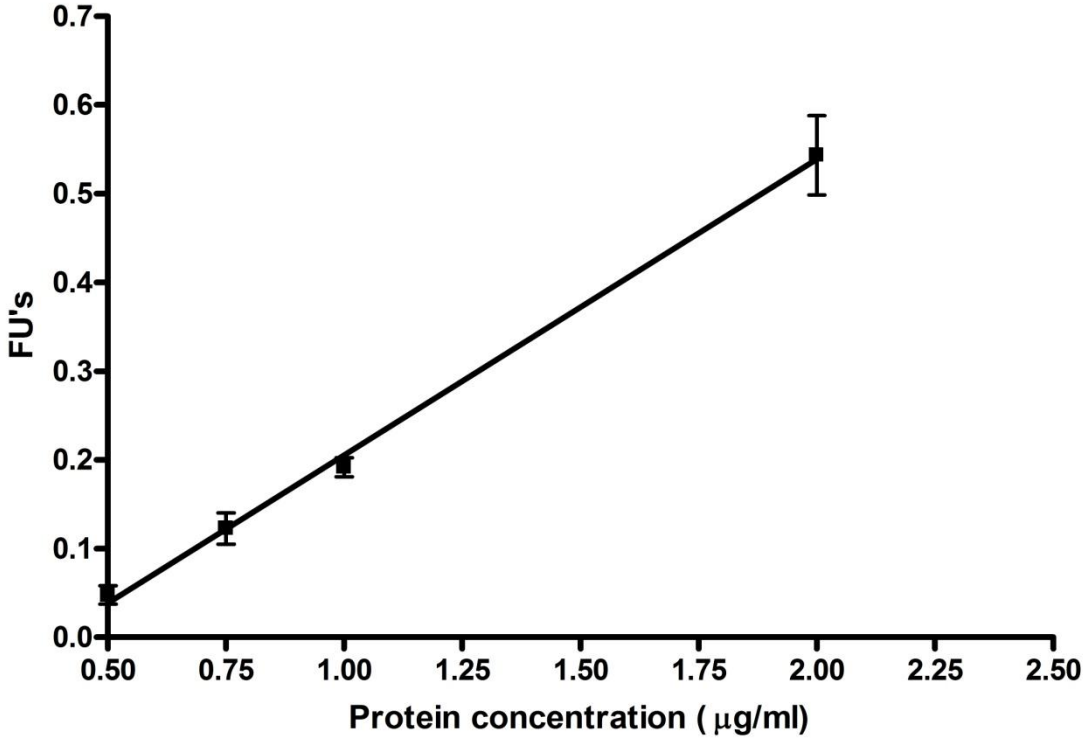


Figure 6-22 Standard curve of BSA scanned using EFSCAN. BSA of differing concentrations was added to 316 stainless steel discs and scanned. An increase in BSA protein concentration results in a linear increase in fluorescence. Data shown is the mean of 6 discs ± the StEM.

Table 6-1 Protein detected on HP parts using EFSCAN analysis. The table also contains protein previously isolated from HP parts using the method described in section 5.2.25 for comparison.

Handpiece Part	Median Protein Concentration detected (µg/part)	Range Protein Concentration detected (µg/part)	Median Protein Previously Detected by SDS extraction and the OPA assay (µg / part)
High Speed Turbine Used, unprocessed	10.17 (n=3)	7.7 – 13.56	1.3 (n=20)
High Speed Turbine Decontaminated	15.75 (n=3)	13.2 – 20.54	0 (n=20)
High Speed Turbines Repair Facility	10.43 (n=3)	5.7 – 18.01	3.4 (n=20)
High Speed Caps Used, unprocessed	5.2 (n=3)	4.2 – 9.6	Not previously sampled
High Speed Caps Decontaminated	6.2 (n=3)	2.6 – 6.6	Not previously sampled
High Speed Caps New	0 (n=1)	0 – 0	Not previously sampled
Spray channels Used, unprocessed	8.7 (n=3)	4.6 – 11.94	15.41 (n=20)
Spray channels, Decontaminated	2.9 (n=3)	2.3 – 10	0 (n=20)
Spray channels, new	0 (n=3)	0 – 0	0 (n=20)

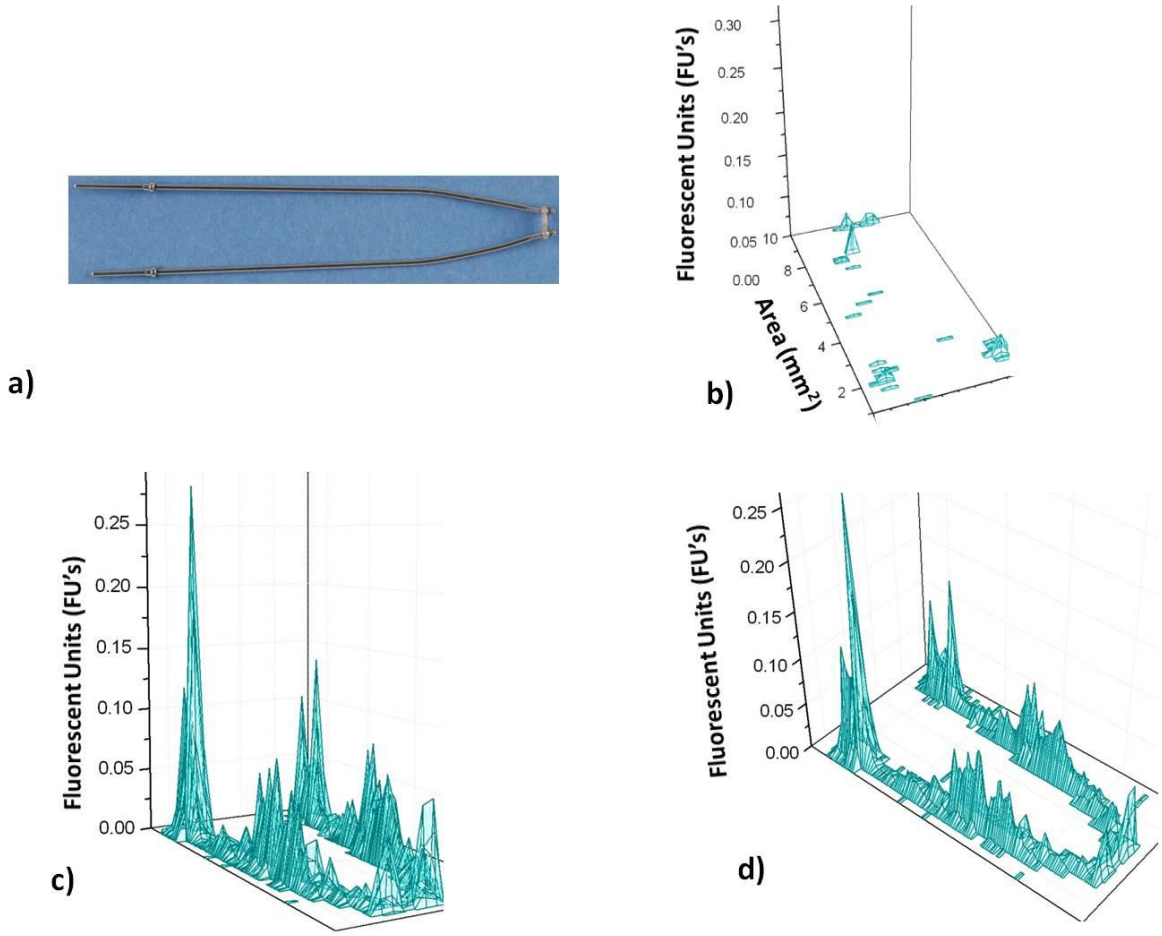


Figure 6-23 EFSCAN analysis of low speed HP spray channel surfaces. Low speed HP spray channels **(a)** that were unused were sampled using EFSCAN analysis **(b).** Spray channels were also sampled after use and before decontamination **(c)** and after decontamination **(d).** Peaks represent protein on the surface. No peaks are visible in the new, unused sample**.** The graphs are representative of 3 spray channel samples.

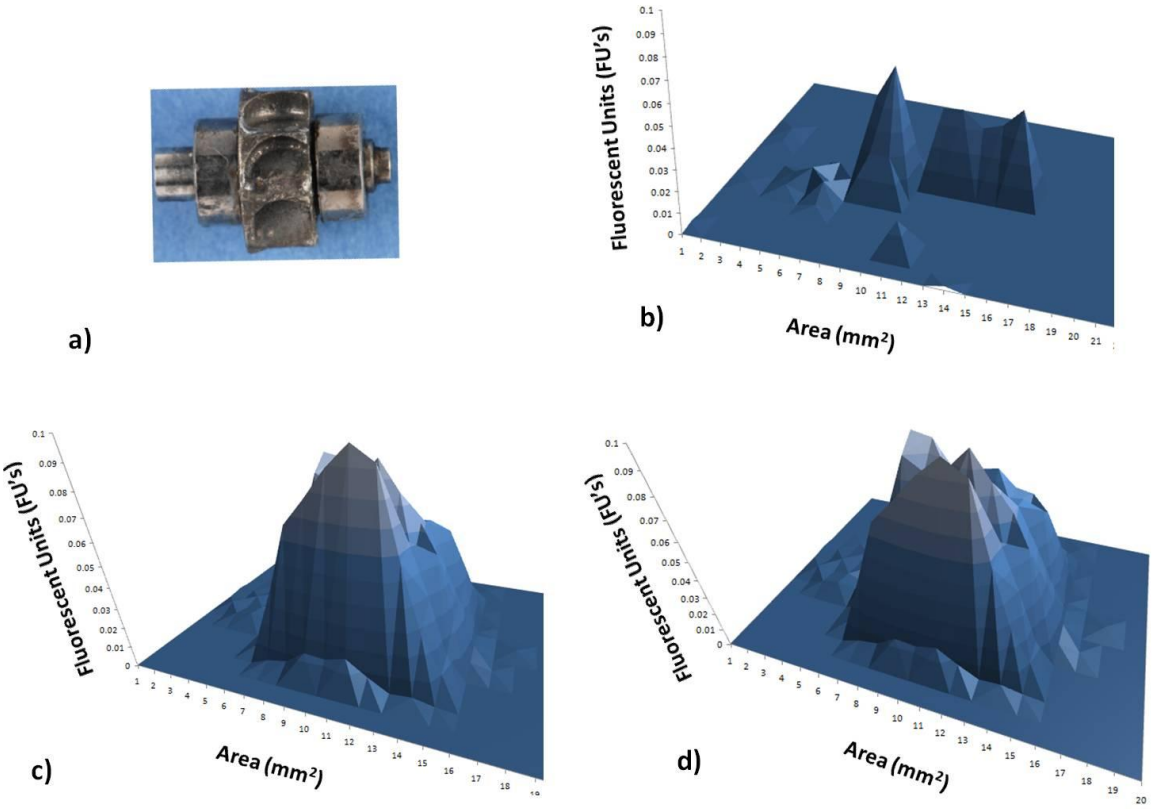


Figure 6-24 EFSCAN analysis of high speed HP turbine surfaces. High speed HP turbines **(a)** that were unused were sampled using EFSCAN analysis **(b).** Turbines were also sampled after use before decontamination **(c)** and after decontamination **(d).** Peaks represent protein on the surface. Significantly lower peaks are visible in the new, unused sample. The graphs are representative of 3 turbine samples.

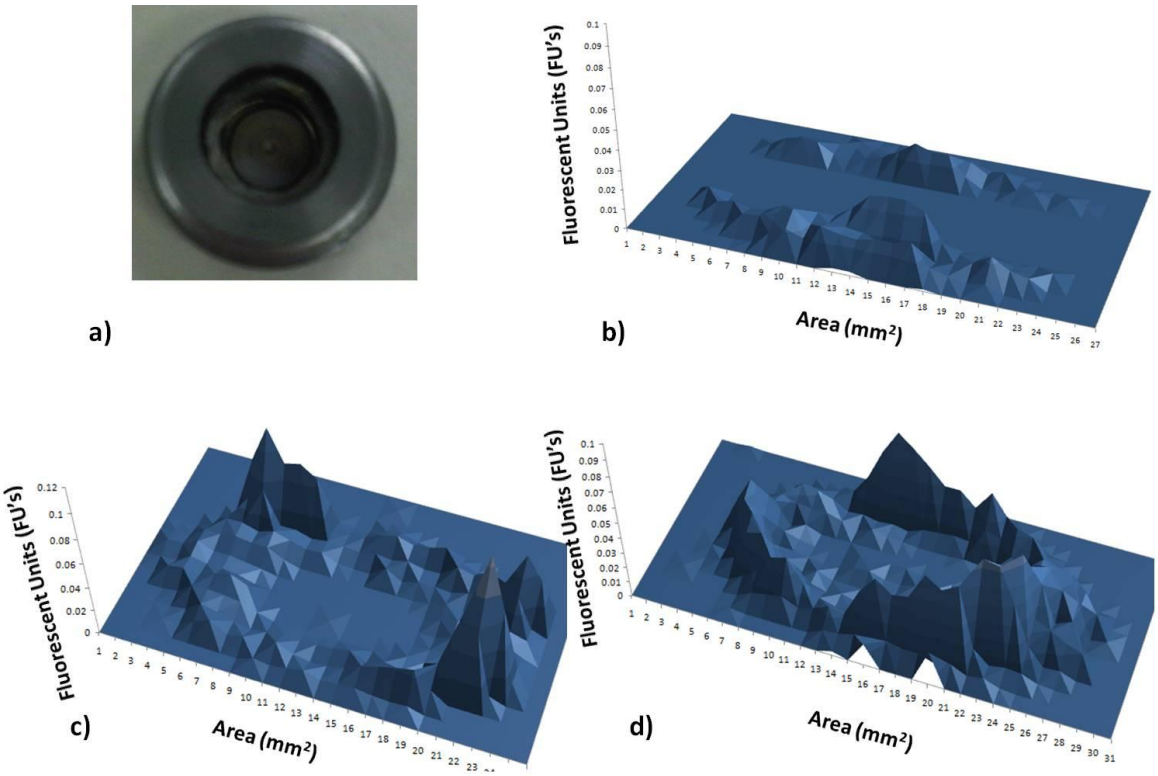


Figure 6-25 EFSCAN analysis of high speed HP cap surfaces. High speed HP caps **(a)** were sampled unused using EFSCAN analysis **(b).** Caps were also sampled after use before decontamination **(c)** and after decontamination **(d).** Peaks represent protein on the surface. Significantly smaller peaks are visible in the new, unused sample **(b).** The graphs are representative of 3 cap samples.

6.2.4 *Discussion*

Methods to determine the efficacy of the cleaning process are under constant validation and review as the cleaning step increases in importance and with increasing concern of the sensitivity of current detection methods (Baxter et al., 2006, Lipscomb et al., 2006a). Surface analysis methods provide an accurate representation of the cleanliness of the surface rather than relying on a visual analysis or desorbing contamination from the instrument surface both of which may have limited sensitivity (Lipscomb et al., 2006b).

To complement the previous work determining the contamination of dental HP parts (Chapter 5, Chapter 6), surface analysis techniques were undertaken to overcome several shortcomings with the previously used methods. These are the characterisation of contaminants that cannot be removed, the identification of additional contaminants that were not previously sampled and to determine the specific locations of contaminants. Surface analysis was also conducted on HP parts that had not been previously sampled. The high speed HP cap has been found to be contaminated through negative pressure along with the turbine and the air and water lines (Matsuyama et al., 1997). The high speed HP caps had not previously been sampled as the caps were impossible to immerse without contacting the outer end of the cap which may be exposed to environmental contamination not associated with HP use. Surface analysis techniques allowed sampling of the inside of the cap and therefore only contamination through HP use would be visualised. For these reasons, the bottom of the surgical HP lever, which is in contact with the surgical HP gear during use, was also sampled using surface analysis. The survey of HP's was also extended to locations not previously associated with HP contamination including the higher and lower gear of the low speed HP.

SEM analysis can provide images of the surface to a resolution that can identify individual bacteria. Without prior fixation of the instrument part, contamination is difficult to characterise as visibility of SEM samples is reliant on the sample being conductive to the electron beam (Anderson, 1951). Fixation requires exposure of the instrument part including sodium cacodylate and glutaraldehyde which means that the HP part must be sacrificed to view biological material. A

turbine and a higher gear from the repair facility were found to have contamination on the surface. The source of the turbines and gears was unknown and therefore the routine HP decontamination process used was not known. All HP's sent to the repair facility came with documentation indicating that a decontamination process had been undertaken before sending and upon receipt had been sterilized using a vacuum sterilizer. Contamination was noted along the surface of the high speed turbine blade and the ballbearing gear cases. This contamination cannot be quantified or identified and fixation may alter the structure of contaminants. Whilst most of the contaminants examined in detail were the size and morphology of bacilli and cocci bacteria, the smaller contamination coating the surface of the turbine may be proteinacious in nature. The fact that contamination was located in all areas of the HP turbine shows the importance of a cleaning process that can clean the turbine blades but also access the ball bearing gears which are in direct contact with the inner structure of the HP head. Contamination was also located on the low speed higher gear despite there being no similar negative pressure due to the low speed HP being powered by electricity. No study has repeated the experiment of Lewis *et al*. (1992) visualising the entry of blue oil into the internal low speed HP parts (Lewis and Boe, 1992) but studies have shown both the *in vitro* and *in vivo* contamination of low speed HP's therefore showing the movement of contamination into the internal areas of the low speed HP (Chin et al., 2006, Herd et al., 2007). Contamination was found on the smooth surface and the complex ridged parts indicating that surface structure does not affect the adsorption of contamination.

To overcome the problem of contaminant identification, EDX analysis was used to identify the elemental contaminants of gross contamination. Only elements with more outer electrons than carbon can be detected due to the movement of electrons from the outer shell to the inner shell of the atom after the removal of inner shell electrons by the electron beam. Movement of the electron causes an emission of X-rays, which is specific for each atom and can therefore identify each atom in a sample. Contaminants were found on every surface sampled including HP parts that had been through the GDH decontamination process (Section 2.1.14, Table 2.2) and the pattern of contamination was similar to previous instrument studies using EDX to measure instrument contamination with

contamination existing as small pockets (Baxter et al., 2006). The main difference for HP contamination compared to previous studies was the presence of lubricating oil which was spread over much of the surfaces. Lubricating oil is part of routine HP maintenance and controversy exists for the ability of the lubricating oil to trap contaminants and to inhibit the sterilization process (Lewis and Boe, 1992) (Edwardsson et al., 1983). Whilst this method cannot completely identify contaminants, the presence of certain elements such as carbon, sulphur and oxygen may indicate the presence of organic contamination such as proteins (Baxter et al., 2006). It is impossible to use the data of the number of contaminants on each surface to undertake a quantitative measurement of protein. Whilst spots of organic contamination are present on all surfaces, it is unclear if this represents a failure of cleaning processes according to BS-EN-ISO-15883 part 1 or if the contaminants present a cross contamination risk. The SEM and EDX method is useful for showing the location of contaminants on the surfaces and may inform development of HP's if there are particular locations on the surface that are more contaminated than others. This was noted for the high speed HP where more contaminants were present on the ball bearing gear than the turbine blade and the surgical gear where the entire spring was covered in contamination.

A method that can combine surface analysis with quantitative data is through the EFSCAN analysis. By scanning BSA standards dried onto stainless steel surfaces, a standard curve can be constructed similar to that used for quantitative protein assays (Chapter 3). Whilst a linear relationship between protein concentration and FU's was observed, the EFSCAN method was not assessed for sensitivity, specificity, and the ability to calculate the protein concentration of common instrument contaminants as detailed by the FDA bioanalytical standards (Chapter 3). The results of new, unused, decontaminated HP parts showed a reaction with the HP surface including lubricating oil that was not significantly larger than the negative control which partly validates the EFSCAN method for use on HP's.

The sensitivity of the EFSCAN analysis was calculated as 5 µg of BSA protein. The sensitivity of the technique can be improved by the addition of fluorecsin isothiocyanate to the proteins on the surface which bonds to the thiol groups of proteins to give a stronger fluorescent signal (Baxter et al., 2009). This process

was not undertaken with dental HP's to avoid any issue with toxicity upon the return of the HP to the GDH population.

Despite being less sensitive than the OPA assay (Chapter 3), more protein was observed on each HP part than the median protein isolated from separate parts sampled using the methods detailed in chapter 6 though the protein concentration isolated for high speed HP turbines and low speed HP spray channels was within the range of protein isolated in chapter 6. This may be due to the protein that is remaining on the surface that is unable to be desorbed using the methods detailed in chapter 6 and this may indicate that dental HP parts contain more residual protein contamination than had been previously estimated. Each turbine and spray channel was only scanned on one side due to the contamination of the instrument part that may occur from placement on the taped surface, the protein concentration on each instrument part may therefore be underestimated. The reading for the spray channels represent the surface of the spray channel rather than the inside of the narrow lumens and lumens would have to be cut open and sacrificed if the internal contamination was to be characterised. There was little difference in protein contamination of instrument parts that had been subjected to a decontamination process though it was unknown what the starting protein concentration of each instrument was before decontamination.

EFSCAN and EDX analysis has previously been utilised to undertake a quantitative analysis of instrument contamination of ophthalmic micro forceps and stainless steel curved artery forceps before and after decontamination using gas-plasma treatment (Baxter et al., 2009). The starting protein concentration of ophthalmic micro forceps was calculated as 1.8 µg or a total of 13.1 FU's and a total of 30.2 FU's were found on curved artery forceps. These FU's were reduced to 0.030 FU's for ophthalmic micro forceps and 0.017 FU's upon gas plasma decontamination and indicated a removal of over 99% of bound protein from the surface (Baxter et al., 2009). A similar sudy detailing the passage of a dental HP through a decontamination process would allow the measurement of the efficacy of HP decontamination technologies. This would allow a comparison of instrument surfaces allowing knowledge of the initial contamination and the calculation of how much protein has been removed from the surface as well as visualisation of areas that retain more contamination.

Previous studies using surface analysis to characterise decontamination on a surface have involved visualisation using a number of methods including episcopic differential interference contrast/epifluorescence microscopy and SYPRO® Ruby analysis (Lipscomb et al., 2006c), Auger spectroscopy (Kobayashi et al., 2009), Ramen spectroscopy, FT-IR spectroscopy (Kobayashi et al., 2009), and ToF- SIMS (Boyd et al., 2001). These contamination studies incorporate surgical instruments and the validation of cleaning processes in other industries such as the dairy industry (Daufin et al., 1991) and the electronics industry (Martin et al., 1999).

Most of the methods highlighted above rely on the bombardment of a sample with an excitation agent and reading the specific reaction of any contaminants upon excitation. Auger spectroscopy, like EDX analysis, relies upon an electron beam to knock out an inner shell electron which causes the movement of electrons from an outer shell and the emission of an auger electron that is specific to each atom (Hofmann, 1979). Ramen and FT-IR spectroscopy rely on the reaction with infrared light with Ramen spectroscopy measuring the scattering of infrared light by the surface and FT-IR spectroscopy measuring the emission of light upon stimulation of the surface with infrared light (Wallach et al., 1970, Becker and Farrar, 1972) . ToF -SIMS relies on the bombardment of the surface with an ion in an ultrahigh vacuum. This causes the scattering of secondary ions of atoms on the surface. These ions can then be analysed using mass spectrometry by calculating the time for the ions to reach the detector. This “time of flight” is compared to the known “time of flight” of atoms (Fitzgerald and Smith, 1995). Epifluorescence microscopy relies on the detection of SYPRO® Ruby stain which interacts with the amino acids lysine, arginine, and histidine to allow for the visualisation of proteins on the surface with an estimated sensitivity of 85 pg/mm^2 of protein (Lipscomb et al., 2006b, Lopez et al., 2000).

A study into the protein contamination of instruments detected with SYPRO Ruby® after cleaning with an AWD (Lipscomb et al., 2006c). A contamination scale was devised with the maximum protein detected being more than 4.2 µg of protein down to a scale of 0-42 ng of protein based on operator visualisation (Lipscomb et al., 2006c). All instruments sampled, tissue forceps, scissors, towel

clips, needle holders, haemostats, were found to have contamination indexes between 3-4 indicating that the protein observed on the surface was between 0.42 to 4.2 µg which the authors indicated showed a “poor” standard of cleaning (Lipscomb et al., 2006c). Contamination of artheroscopic blades decontaminated using high pressure water flow, ultrasonic cleaning and sterilized using ethylene oxide was measured using FT-IR and Auger spectroscopy (Kobayashi et al., 2009). The study found contaminants including collagen, proteins, hydrocarbons, calcium carbonate, hydroxyapetite, esters, and fatty acids (Kobayashi et al., 2009). Whilst it is clear that contaminants remain on the surface after decontamination, the authors state that the impact of these contaminants on infection control requires further study (Kobayashi et al., 2009). The study also highlighted the more detailed identification of contaminants through FT –IR spectroscopy compared to EDX analysis as only an elemental analysis was possible using EDX (Kobayashi et al., 2009).

Though the ToF SIMS technique has not been used for surgical instrument contamination, the technique has been previously used to determine the cleanability of stainless steel contaminated with milk powder using a brush or a spray cleaning technique (Boyd et al., 2001). The spray cleaning technique was less efficacious at removing fatty acids than the brush technique though the spray technique resulted in an increase in surface protein removal (Boyd et al., 2001). This study shows the potential application of the ToF-SIMS technique to instrument contamination.

These sensitive surface analysis techniques can be used for the validation of cleaning processes. By sampling an instrument before and after decontamination at specific sites where contamination is noted, the ability of the cleaning process to remove these contaminants can be assessed. The definition of “clean” may require readjustment as the increase in sensitivity of detection afforded by these techniques allows for the detection of smaller concentrations of protein and the cross contamination risk would have to be assessed. A combination of quantitative surface analysis methods such as EFSCAN and qualitative methods allowing for identification of these contaminants may help in this decision.

In conclusion, contamination was detected in HP parts before and after decontamination processes. A study into the cleanability of HP's using documented processes should be considered to note the effect the cleaning process has on HP contamination. There exists a variety of surface analysis techniques to provide a microscopic view and detailed identification of instrument surface contaminants. In conjunction with previously described techniques to desorb contamination from the surface (Chapter 4, Chapter 5), it is possible to build a complete picture of instrument surface contamination. Whilst it is possible to examine groups of used, unprocessed and decontaminated instruments, the techniques are best used as part of validation of cleaning processes to put contamination into context and to identify areas where contamination remains to help inform development of instrument design or the cleaning process (Baxter et al., 2009). Qualitative data can also help put contaminants into context by determining any cross contamination risk or the study. The increased sensitivity of the techniques may result in more "failures" of the cleaning process and defining an acceptable standard when considering sensitive surface analysis techniques should depend on the nature of the contaminant (Lipscomb et al., 2006c).

7 Chapter 7: Assesment of handpiece cleaning processes, chemicals and equipment.

7.1 Introduction

The cleaning stage of the instrument decontamination process is used to remove inorganic and organic debris that may inhibit the subsequent disinfection and sterilization processes. These contaminants can be as diverse as proteins and complex bacterial biofilms. From the previous work in this study, it is clear that HP's are contaminated with bacteria and protein associated with blood contamination (Chapter 4, Chapter 5).

The first stage of biofouling of medical instrumentation is the adsorption of protein on the surface (Desroches et al., 2007). Bacteria can then adsorb to the surface using specific receptors for human proteins such as fibrinogen (Tegoilia and Cooper, 2002). When bacterial cells adsorb to a surface, cells can form a matrix consisting of polysaccharides, protein nucleic acids and amphiphilic compounds known as a biofilm (Costerton et al., 1978). The biofilm structure provides a physical and chemical barrier for the bacterial cells against antimicrobial agents and can also inhibit sterilization processes (Campanac et al., 2002, Gibson et al., 1999). A biofilm also allows the retention and shedding of bacterial cells if untreated. Cleaning solutions allow disruption of the biofilm structure and removal of adsorbed protein (Gibson et al., 1999).

The parameters that affect cleaning efficacy are summarised in the Sinners circle, and these include the temperature of the cleaning solution, the cleaning solution used, the amount of time that cleaning occurs for, and the amount of mechanical energy that is used for the cleaning process (Smulders et al., 2007). Changing a variable may have a detrimental impact on cleaning outcomes or may result in more efficacious cleaning.

Whilst most variables can only increase or decrease, changing the cleaning solution and the water quality may have multiple affects on the cleaning process. Detergents often have complex and unknown formulations and have different active compounds. Concerns have been raised over the effect of certain cleaning solutions on contaminant removal with some studies demonstrating the fixing of blood and protein to surfaces (Prior et al., 2004, Nakata et al., 2007). The water quality may also affect the cleaning process with

dissolved solids and the hardness of the water having an effect on cleaning efficacy. The parameters of water are dependent on the water source and may impact on the efficacy of cleaning at different locations.

Previous work into cleaning efficacy has utilised either the *in vivo* process or involved the development of models based on various cleaning parameters. For some complex models, it is difficult to determine what effect the cleaning solution is having on cleaning efficacy (Alfa et al., 2006). A simpler cleaning model can measure the direct effects of the detergent on the surface to justify the expense of including them in a cleaning process.

For washer disinfector cleaning validation, the European standard BS- EN- ISO-15883 parts 1-5 provide details on the construction of test devices and the application of test soils to assess the efficacy of the washing processes. The test soil applied is dependent on the instruments or materials to be reprocessed and vary from defibrinated horse blood to complex formulations containing flour, blood and bacteria (Zuhlsdorf and Martiny, 2005). The efficacy of the cleaning process is measured using of 1 of 3 protein assays after desorption of residual protein from the test pieces or instruments.

The aim of this chapter was to first develop and utilise simple *in-vitro* cleaning models to study the effect of alteration of cleaning parameters on the efficacy of blood removal from a stainless steel surface including the sampling of cleaning solutions recommended by the dental clinical effectiveness program. These detergents were then assessed for the ability to disrupt biofilms formed by dental HP isolates *in vitro*. The cleaning efficacy of novel HP cleaning machines was also assessed through the application of a protein based test soil on 3 different HP's that was based on typical HP protein contaminants (Chapter 5).

7.2 Results

7.2.1 *Water quality and detergent properties*

Tap water was obtained from the Glasgow Dental Hospital on the day of the experiment. The water and detergent qualities were measured using a PCSTestr 35 (Eutech instruments Nijkerk Holland). The pH, conductivity, salinity and total dissolved solids of each water sample were determined (Table 7-1). A total of 3 alkaline detergents, 3 enzymatic detergents, a handwash and a dental HP cleaning solution were sampled in the experiment along with 0.1M NaOH (Table 7-2).

Table 7-1 Properties of H_2O used in the study

H_2O source	pH	Conductivity (µS)	Salinity (ppm)	Total dissolved solids (ppm)
Tap (n=3)	6.41	63.7	35.6	46.2
Reverse Osmosis(n=3)	5.49	2.3	12.5	3.6

Table 7-2 Parameters of sampled detergents

Detergent	Manual	Ultra Sonic	AWD	Dosage	Temp Specified	pH
Alconox®	Yes	Yes	No	10g detergent/1l H2O	H2O = cold, warm or hot	9.5
Endozime® AW+®	Yes	Yes	Yes	34ml detergent/8l H2O	H_2O = warm	7.13
Haemo-sol®	Yes	Yes	No	20g detergent/ 5l H2O	H_2O = warm (50˚C)	10.5
HiBi® Scrub	N/A	N/A	N/A	N/A	N/A	5
Rapidex®	N/A	N/A	N/A	1 sachet (28g) detergent	H_2O = 50˚C (20˚C-70˚C acceptable)	11
Rapizyme®	Yes	Yes	Yes	10ml detergent/ 5l H2O	H_20 = 38˚C	7.24
Sonozyme®	No	Yes	No	1 sachet (25ml)/ 8l H2O	N/A	5.5
0.1M NaOH	N/A	N/A	N/A	N/A	N/A	9.3

N/A Denotes no instructions for use in machines or in cleaning processes.

7.2.2 *Effect of cleaning time on blood removal from stainless steel discs*

The effect of agitation time on blood removal was assessed by setting the rocking platform to a moderate speed of 25, 20° tilts/min and 100 µl samples were taken at 1, 5, 10, 15, and 20 min. Samples were also taken of negative control discs at each time point. The experiment consisted of 3 discs and the experiment repeated 3 times. For statistical analysis, data was compared using a one way analysis of variance (ANOVA).

A median total protein concentration of 3.2 mg was added to each disc. An increase in time resulted in an increase in blood removal (Figure 7-1,Table 7-3 Blood removal from a stainless steel surface with time). A median of 201 µg of protein was isolated after 1 min of agitation, 528 µg after 5 min agitation, 1963 µg after 10 min agitation, 2681 µg after 15 min agitation and 3211 µg after 20 min agitation. Significantly ($p<0.05$) more blood is removed between 5 and 10 min though no significant difference is observed between the other time points. Over 90 % of blood protein is removed after 20 min of cleaning. No protein was isolated from the negative control discs.

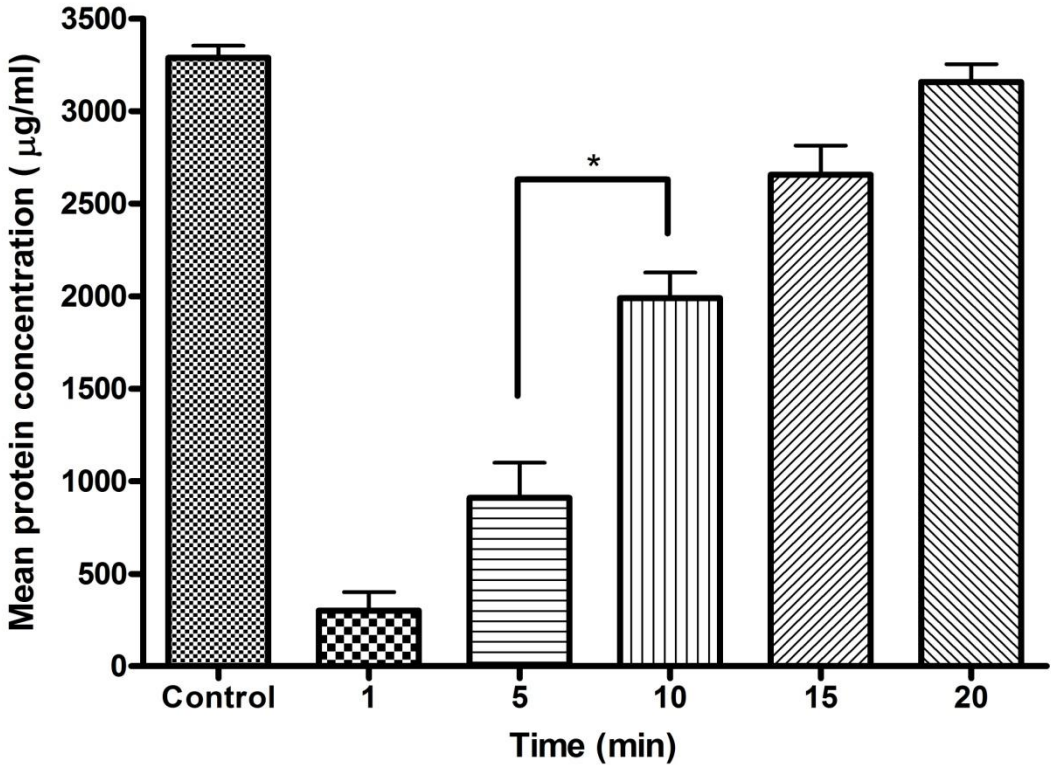


Figure 7-1 Effect of cleaning time on the removal of citrated blood from a stainless steel surface. The effect of cleaning time on blood removal from a stainless steel surface was assessed by measuring the protein desorbed using the BCA assay. The agitation speed was kept constant at 25 20°C tilts/min and was assessed at ambient room temperature (22°C). An increase in cleaning time results in an increase in protein removal from the surface with significantly (* = $p<0.05$) more blood removal observed between 5 to 10 min. The data shown is the mean of results from, 3 discs from 3 experiments and the StEM. The control shows the initial blood protein concentration applied to the stainless steel disc.

Table 7-3 Blood removal from a stainless steel surface with time

Time (min)	Mean % Blood removed	Median Blood Protein Removed (µg)	Range Blood Protein Removed (µg)
1	13	201	0 – 1044
5	26	528	362.4 – 1875
10	55	1963	1032 – 2676
15	80	2681	1976 – 3348
20	94	3211	2593 – 3411
Positive control		3235	3190 – 3421
Negative control		<5	<5

7.2.3 *Effect of reverse osmosis water temperature on the removal of blood from a stainless steel disc*

For the effect of ROH_2O temperature on blood removal, the tilting platform was set to 25, 20°tilts/min and each well containing a disc was inoculated with 1 ml of RO H_2O at the temperatures of 22°C, 38°C and 50°C. The tilting platform was inserted into a KS40001 incubator (IKA®, Staufen Germany) at the appropriate temperature and a 100 µl sample was taken after 5 min. Samples were also taken of negative control discs for each temperature. The experiment consisted of 3 discs for each temperature of water sampled. For statistical analysis, data was compared using a one way ANOVA.

A median of 4.2 mg of protein was added to each stainless steel disc. An increase in ROH_2O temperature results in an increase in blood removal (Figure 7-2,Table 7-4). A median of 1638 µg (range 915 – 1904 µg) of protein was removed after 5 min when the ROH_2O temperature was 22°C , 2155 µg (range 1387 – 2996 µg) of protein was removed at a temperature of 37°C and a median of 2656 µg (range 1328 – 3778) was removed at a temperature of 50°C Significantly ($p<0.01$) more blood is removed when the RO H2O temperature is raised from 22°C to 50°C (Figure 7-2,Table 7-4). No significant difference was noted when the RO H_2O temperature was raised from 22°C to 38°C. No protein was isolated from the negative controls.

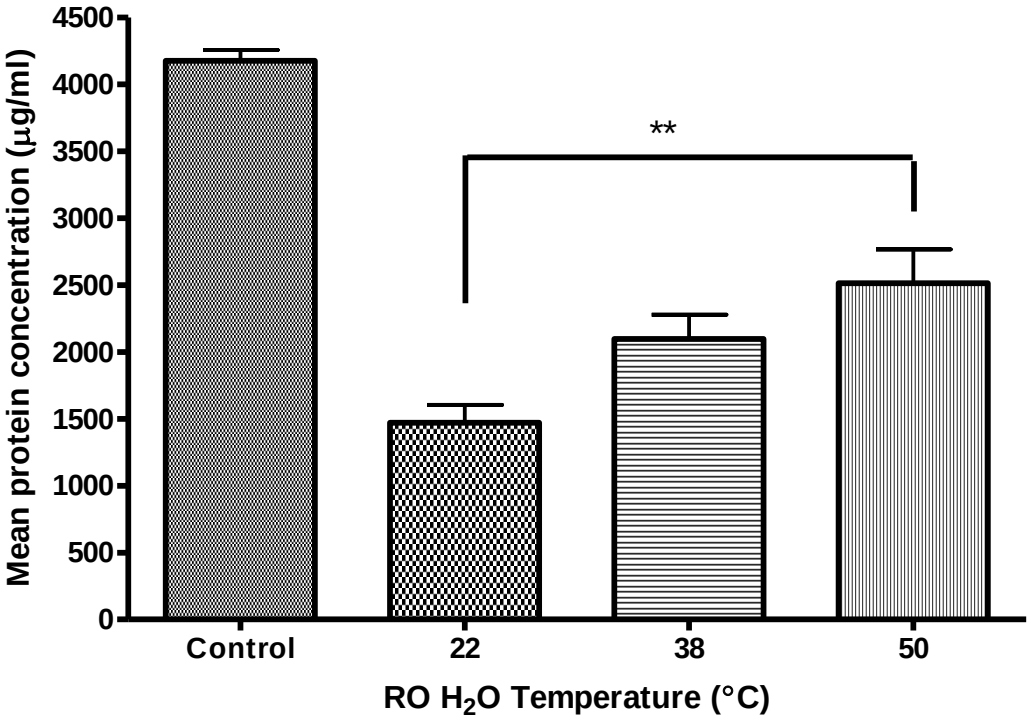


Figure 7-2 Effect of water temperature on the removal of citrated blood from a stainless steel surface The effect of ROH_2O temperature on blood removal from a stainless steel surface was assessed by measuring the protein desorbed using the BCA assay. The agitation speed was kept constant at 25 tilts/min at 20°C and samples were taken after 5 min. An increase in cleaning time results in an increase in protein removal from the surface with significantly (* = $p<0.05$) more blood removal observed between 5- 10 min. The data shown is the mean of results from, 3 discs from 3 experiments and the StEM. The control shows the initial blood protein concentration applied to the stainless steel disc

Table 7-4 Effect of ROH$_2$O temperature on the percentage of blood removal

RO H_2O Temperature	Mean % Blood Removed	Median Blood Protein Removed (µg)	Range Blood Protein Removed (µg)
22˚C	35	1638	915- 1904
37˚C	50	2155	1387 – 2996
50˚C	60	2656	1328 – 3778
Positive Control		4029	4029 – 4309
Negative Control		<5	<5

7.2.4 *Effect of agitation speed on the removal of blood from a stainless steel disc*

Each well containing a disc was immersed in 1 ml of RO H_2O. To assess the effect of agitation speed, the rocking platform was set at 0, 25, and 45, 20° tilts/min and 100 µl samples were taken at 5 min. Samples were also taken of negative control discs for each agitation speed. The experiment consisted of 3 discs for every agitation speed sampled and the experiment repeated 3 times. For statistical analysis, data was compared using a one way ANOVA.

A median of 3.1 mg of protein was added to each stainless steel disc. An increase in agitation speed results in an increase in blood removal (Figure 7-3,

Table 7-5). A median of 374 µg (range 80 – 765 µg) of protein was removed when no agitation speed was applied, a median of 1158 µg (range 628.5- 1567 µg) was removed when a 25 tilts/min agitation speed was applied and a median of 2448 µg (range 1508 – 2818 µg). Increasing the agitation speed to 25 tilts/min results in significantly ($p<0.05$) more blood removal than when no agitation speed was applied and significantly ($p<0.001$) more blood is removed when the agitation speed is increased from 25 tilts/min to 45 tilts/min. No protein was isolated from the negative controls.

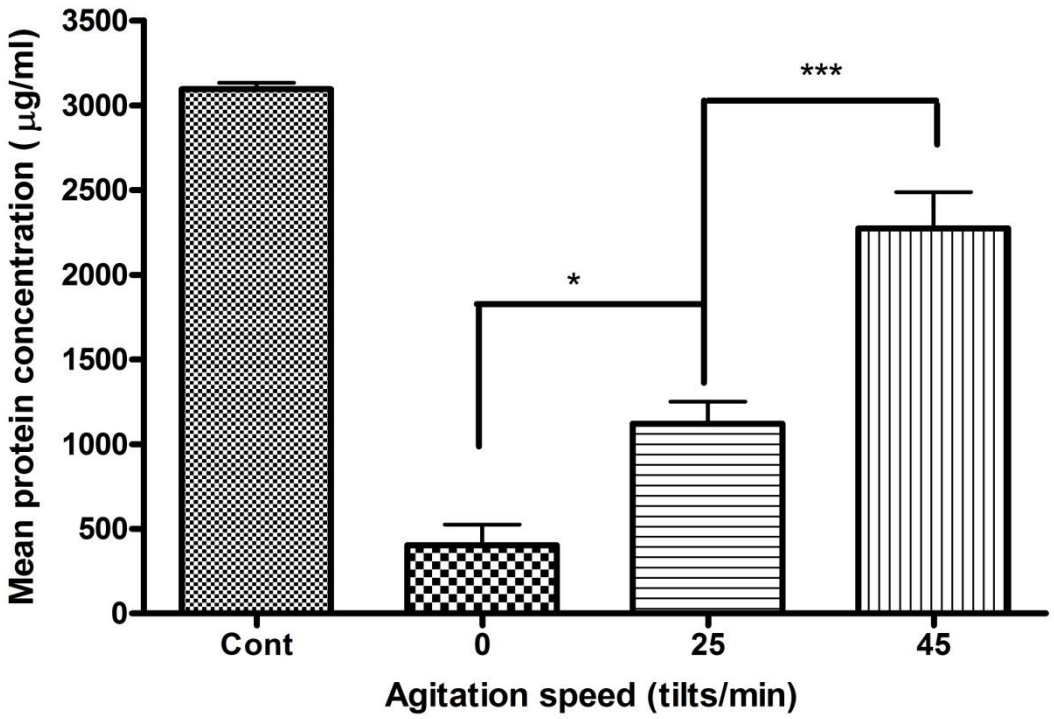


Figure 7-3 Effect of agitation speed on the removal of citrated blood from a stainless steel surface. The effect of agitation speed on blood removal from a stainless steel surface was assessed by measuring the protein desorbed using the BCA assay. Samples were taken after 5 min and assessed at ambient room temperature (22°C) An increase in agitation speed results in an increase in protein removal from the surface with significantly (* = $p<0.05$) more blood removal observed when the speed is increased from 0 to 25 tilts/min and when the speed is increased from 25 tilts/ min to 45 tilts/ min (***$p<0.001$). The data shown is the mean of results from, 3 discs from 3 experiments and the StEM. The control shows the initial blood protein concentration applied to the stainless steel disc.

Table 7-5 Effect of agitation speed on the percentage and median blood removal from a stainless steel surface.

Agitation Speed	Mean % Blood Protein Removed	Median Blood Protein Removed (µg)	Range Blood Protein Removed (µg)
0	13	374	80 – 765
25	36	1158	628 – 1567
45	73	2448	1508 – 2818

7.2.5 *Efficacy of blood removal by detergents at room temperature*

Each sampled detergent was diluted according to manufacturer's instructions in RO H_2O and the cleaning parameters were set at a room temperature solution of 22 ^{O}C with the tilting platform set to 25, 20° tilts/min and a 100 µl sample taken after 5 min. Detergents were also assessed at the manufacturers recommended temperatures and compared to RO H_2O at the same temperatures. For statistical analysis, the protein removed by each detergent was compared to the protein removed by tap H_2O using a one way ANOVA.

A median of 3.7 mg of blood protein was added to each stainless steel disc. The total blood protein removed by 9 cleaning solutions was compared with total blood protein removal by tap H_2O alone (Figure 7-4). For each experiment, ROH_2O removal of blood was also assessed at the same time. No solutions removed significantly more blood than the ROH_2O control which removed a median of 1421 µg of protein (range 516- 1901 µg) including 0.1M NaOH which removed a median of 1691 µg (range 1170 – 2183 µg). Of all the cleaning solutions sampled, Haemo-sol® removed the most blood protein with a median of 2070 µg (range 1314 – 3624 µg) removed (Figure 7-5). HiBi Scrub® removed significantly ($p<0.001$) less blood protein with a median of 0 µg (range 0 – 641 µg) than ROH_2O (Figure 7-5). For the alkaline detergents, Alconox® removed the least amount of blood protein of the detergents with a median of 927 µg (range 137 – 1730 µg) and Rapidex® removed a median of 1585 µg (range 1020 – 2405) of blood protein (Figure 7-5). Of the enzymatic detergents, Sonozyme® removed the most blood protein with a median of 1907 µg (range 1248 – 2754) Rapizyme® removed a median of 975 µg (range 245 – 2816 µg) of blood protein and Endozime® detergent removed a median of 1421 µg (range 694 – 1984 µg). Tap H_2O removed less blood protein than ROH_2O with a median of 1356 µg (range 845 – 2176) removed.

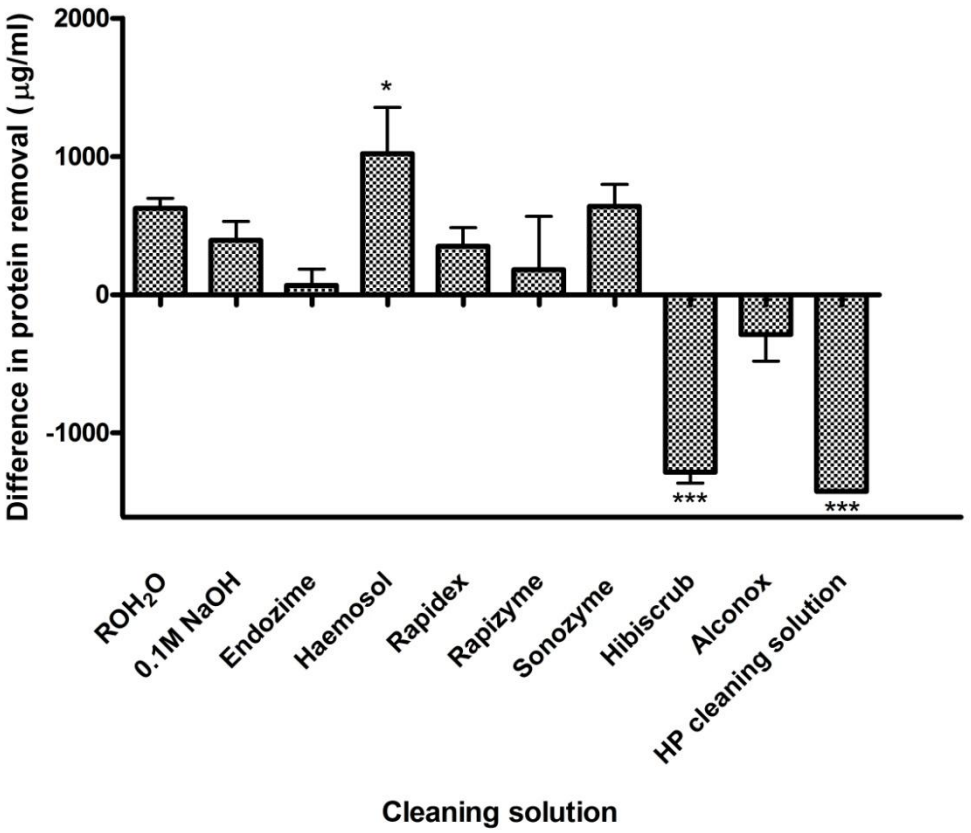


Figure 7-4 Comparison of blood protein removal by cleaning solutions. The efficacy of cleaning solutions at removing blood removal from SSS's after 5 min of cleaning was assessed by measuring the protein desorbed using the BCA assay. All cleaning solutions were compared to blood removal by tap H_2O which is represented by the baseline of the graph. Samples were assessed at ambient room temperature (22˚C) and the agitation speed was set at 25 20˚C tilts/min. All cleaning solutions were compared to the blood protein removed by tap H_2O. No cleaning solutions removed significantly more blood than ROH_2O alone though HiBi® Scrub and W & H cleaning solution remove significantly (***= $p<0.001$) less protein. Haemo-sol® detergent removed the most blood protein of the cleaning solutions sampled. Tap H_2O removed less protein than RO H_2O though this was not significant. The data shown is the mean of results from, 3 discs from 3 experiments and the StEM.

Efficacy of detergents at blood removal at manufacturers recommended temperature

Each sampled detergent was diluted according to manufacturer's instructions in RO H_2O and the tilting platform set to 25, 20° tilts/min and a 100 µl sample taken after 5 min. Detergents were also assessed at the manufacturers recommended temperatures and compared to RO H_2O at the same temperatures. For statistical analysis, the protein removed by each detergent at manufacturers recommended temperature was compared to protein removed by detergents at room temperature using a one way ANOVA.

A median of 3.1 mg of protein was applied to each stainless steel disc at 22°C and 50°C. When the environmental and the ROH_2O temperature was increased to 50°C, ROH_2O removed a median of 2761 µg (range 2296 – 3000 µg) blood protein compared to a median of 1380 µg (range 0 – 1901 µg) blood protein at 22°C. Alconox® detergent removed a median of 1817 µg (range 798 – 2296 µg) blood protein compared to a median of 927 µg (range 137 – 1731 µg) of blood protein at 22°C. Haemo-sol® detergent removed a median of 2401 µg (range 1622 – 2776 µg) blood protein compared to a median of 2070 µg (range 1314 – 3024 µg) of blood protein at 22°C (Figure 7-5,Table 7-6). Rapidex® detergent removed a median of 2476 µg (range 1428 – 3375 µg) blood protein compared to a median of 1585 µg (range 1020 – 2405 µg) of blood protein at 22°C (Figure 7-5,Table 7-6). Rapidex® detergent removed significantly ($p<0.05$) more blood protein at 50°C than at 22°C (Figure 7-5,Table 7-6). A significant ($p < 0.01$) increase in percentage blood removal also occurred in ROH_2O alone when the temperature was increased (Figure 7-5,Table 7-6). Alconox® and Haemo-sol® removed more blood protein at 50°C compared to 22°C though no significant difference was noted (Figure 7-5,Table 7-6). No protein was isolated from the negative controls.

When the environmental and solution temperature was raised to 38°C, RO H_2O removed a median of 2155 µg (range 1387 – 2996 µg) of blood protein compared to a median of 1380 µg (range <5 – 1901 µg) (Figure 7-6,Table 7-7). Endozime® detergent removed a median of 1893 µg (range 798 – 2296 µg) blood protein compared to a median of 1421 µg (range 137 – 1731 µg) of blood protein at 22°C (Figure 7-6,Table 7-7). Rapizyme® detergent removed a median of 2311 µg

(range 1879 – 3050 µg) blood protein compared to a median of 975 µg (range 245 – 2581 µg) of blood protein at 22°C (Figure 7-6, Table 7.12). Endozime® removed significantly ($p<0.001$) more blood protein at 38°C than 22°C (Figure 7-6,Table 7-7). No significant difference in percentage blood removal was noted between Rapizyme® and RO H_2O at both temperatures (Figure 7-6,Table 7-7).

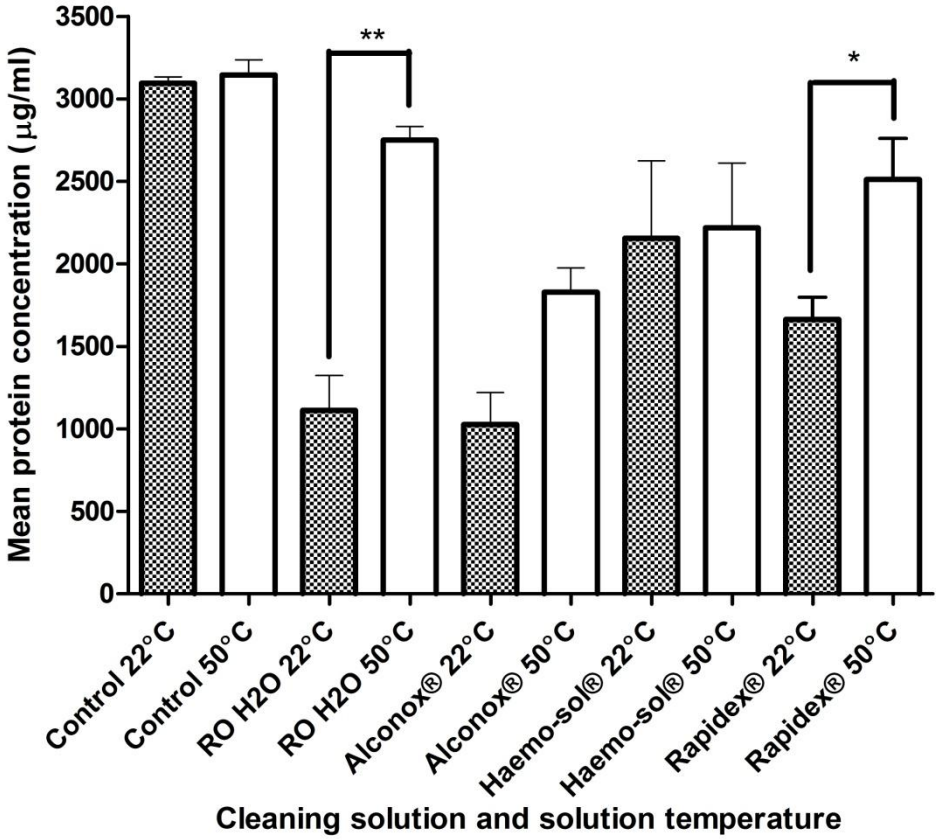


Figure 7-5 Effect of detergent manufacturers suggested temperature on the percentage blood removal by detergents. The efficacy of detergents at blood removal from stainless steel surfaces after 5 min cleaning at room temperature (grey bars) and 50°C (white bars) with the agitation speed set to 25 20°C tilts/min was assessed by measuring the protein desorbed using the BCA assay. Raising the solution and environmental temperature to 50 °C resulted in a significant increase (*= $p<0.05$, ** = $p<0.01$) in ROH_2O blood removal and Rapidex® blood removal. Alconox® blood removal was increased but not significantly. No difference was observed in the case of Haemo-sol® detergent. The data shown is the mean of results from, 3 discs from 3 experiments and the StEM. The control shows the initial blood protein concentration applied to the stainless steel discs at each temperature.

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Table 7-6 Mean percentage and protein removed by each detergent at 22˚C and 50˚C

Cleaning Solution	**Mean % Blood removed**	**Median Blood Protein Removed (µg)**	**Range Blood Protein Removed (µg)**
RO H_2O 22˚C	36	1380	<5 – 1901
RO H_2O 50˚C	87	2761	2296 – 3000
Alconox® 22˚C	33	927	137 – 1731
Alconox® 50˚C	58	1817	799 – 2296
Haemo-sol® 22˚C	71	2070	1314 – 3624
Haemo-sol® 50˚C	71	2401	1622 – 2776
Rapidex® 22˚C	53	1585	1020 – 2405
Rapidex® 50˚C	82	2476	1428 – 3375
Positive Control 22˚C		3104	3023 -3157
Positive Control 50˚C		3180	2970 – 3284
Negative Control 22˚C		<5	<5
Negative Control 50˚C		<5	<5

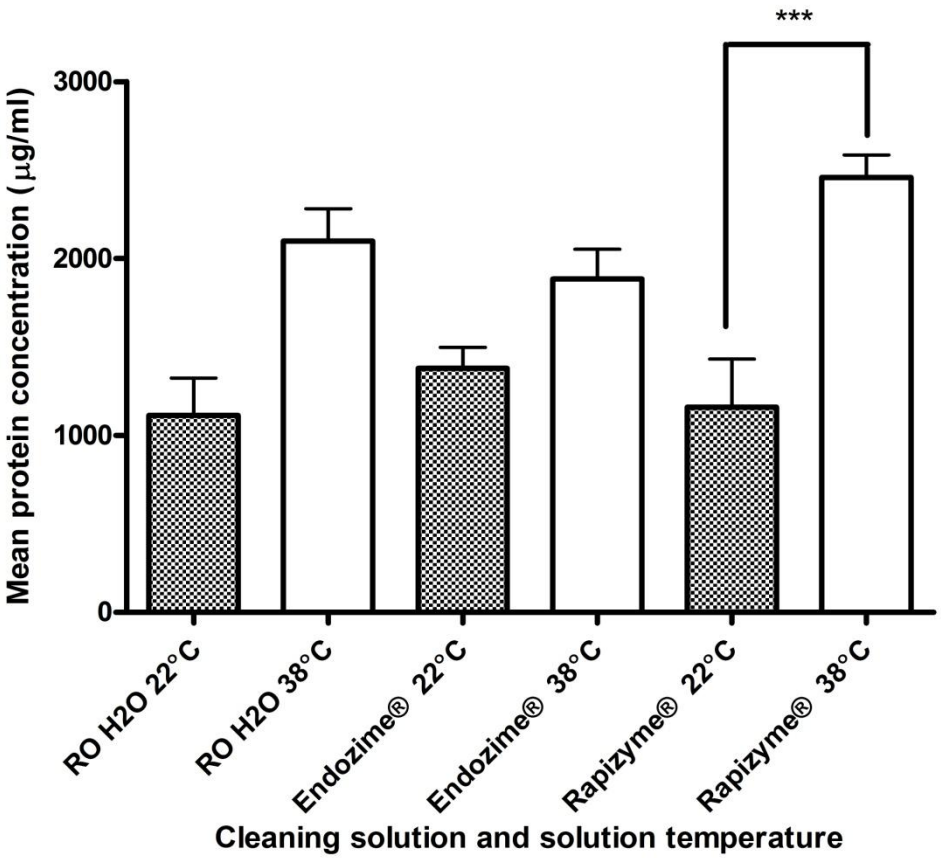

Figure 7-6 Effect of detergent manufacturers suggested temperature on the percentage blood removal by detergents. The efficacy of detergents at blood removal from stainless steel surfaces after 5 min cleaning at room temperature (grey bars) and 38˚C (white bars) with the agitation speed set to 25 20˚C tilts/min was assessed by measuring the protein desorbed using the BCA assay. Raising the solution and environmental temperature to 38˚C resulted in a significant increase (*** = $p<0.001$) in Rapizyme® blood removal. ROH_2O and Endozime® blood removal was increased but not significantly. The data shown is the mean of results from 3 discs from 3 experiments and the SEM. The control shows the initial blood protein concentration applied to the stainless steel discs at each temperature.

Table 7-7 Mean percentage and protein removed by each detergent at 22˚C and 38˚C

Cleaning Solution	Mean % Blood removed	Median Blood Protein Removed (µg)	Range Blood Protein Removed (µg)
RO H_2O 22˚C	36	1380	<5 – 1901
RO H_2O 38˚C	79	2155	1378 – 2996
Endozime® 22˚C	45	1421	137 – 1731
Endozime® 38˚C	58	1893	798 – 2296
Rapizyme® 22˚C	37	975	245 – 2581
Rapizyme® 38˚C	75	2311	1879 – 3050
Positive Control 22˚C		3104	3023 -3157
Positive Control 50˚C		3161	3063 – 3551
Negative Control 22˚C		<5	<5
Negative Control 50˚C		<5	<5

7.2.6 *Efficacy of detergents at P. acnes biofilm removal*

A total of 20 *P. acnes* isoates including 1 1A types, 10 1B types isolated from GDH HP's (Section 4.2.4) and 19 *P. acnes* isolates including 3 IA types, 8 1B types and 1 II type isolated from HP's from the St Albans repair facility (Section 4.2.5) were resurrected from Pro-tect beads. A single bead was plated onto FAA agar and incubated anaerobically for 24 h at 37°C. *Staphylococcus epidermidis* strain was cultured onto blood agar and incubated overnight at 37 °C under 5% CO_2. A single colony of each *P. acnes* strain and the *S. epidermidis* was added to 50 ml of reinforced clostridial (RC) broth that had been pre-reduced by boiling for 30 min. Of the GDH isolates, a total of 13 cultures were incubated overnight at 37°C anaerobically and 7 cultures were incubated overnight aerobically at 37°C. Of the repair facility isolates a total of 11 cultures were incubated overnight at 37°C anaerobically and 8 cultures were incubated overnight at 37°C aerobically.

The optical density (OD_{460}) of each culture was taken using a spectrophotometer (Fisherbrand). Each culture was diluted in RC broth to an O.D of 0.2 representing $1x10^8$ cfu of bacteria. A total of 6, 300 µl samples of each isolate were added to separate wells of a clear Costar 96 well flat bottomed plate (Sigma Aldrich Dorset UK). Separate plates were used for each cleaning solution. Each plate was placed on a PMR tilting platform (Grant Instruments Cambridge, UK) at moderate speed (18 RPM) and incubated for 72 h anaerobically at 37°C or for 16h aerobically at 37°C. RC media was added to each plate as a negative control. RC broth was changed every 24h in each well for the 72h biofilms.

For 72h biofilms, a 1% (v/v) solution of Haemo-sol® detergent and undiluted W & H cleaning solution were sampled. For 16h biofilms, a 1% (v/v) solution of Alconox®, Haemo-sol® and Rapizyme® and an undiluted W & H cleaning solution were samped. The media was removed from each well PBS and a 300 µl sample of the appropriate cleaning solution was added to each well and incubated at room temperature for 16 min which is the length of time an instrument is exposed to detergent during some automated washing process (Section 2.1.3). After exposure, the supernatant was removed and the wells rinsed a further 3 times in sterile PBS. Samples were compared to a positive, untreated control

and a positive control treated with PBS in place of a cleaning solution. The media was removed each day and cultured on FAA plates as controls.

For biomass sampling, a total of 300 µl of 1% (w/v) crystal violet solution was added to each well and incubated at room temperature for 10 min. The wells were rinsed 3 times by tap water and 300 µl of a 70 % ethanol/ 5 % acetone solution was added to each well. The plate was incubated for 15 min and the solution transferred to a fresh plate before being read in a plate reader (Sunrise™ Tecan) at OD_{570}. The bacterial cell viability of 16h biofilms was measured using the alamarBlue® assay (Invitrogen Paisley UK) according to manufacturer instructions. Each plate was incubated for 4 hours aerobically at 37 °C and the plate measured in an omega flurostar plate reader at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

A total of 23/28 *P. acnes* isolates sampled were formed biofilms under 72h anaerobic conditions and a total of 13/15 isolates sampled formed biofilms under 16h aerobic conditions with the biomass resulting in a crystal violet OD_{570} reading significantly greater than the negative control (Holmberg et al., 2009). After growth for 72 h and upon exposure to cleaning solutions, Haemo-sol® detergent significantly ($p < 0.001$) reduced the *P. acnes* biofilm biomass to a median of 0.6 (range 0.4 – 1.2) compared to exposure to RO H_2O where the biomass was a median of 0.9 (range 0.4 – 1.6) (Figure 7-7). Exposure to W &H cleaning solution resulted in a biomass median of 0.9 (range 0.5 – 1.507) (Figure 7-7). After 72h growth of the *S. epidermidis* RP62A strain the biofilm biomass was calculated as a median OD_{570} of 1.1 (range 0.5 – 2) after exposure to PBS when the data from all experiments was combined (n=18) (Figure 7-8). Haemo-sol® significantly ($p<0.05$) reduced the biomass to a median OD_{570} of 0.4 (range 0.2 -1.6) compared to exposure to ROH_2O which upon exposure reduced the biomass to a median of 0.78 (range 0.4 – 1.8) (Figure 7-8). No significant reduction of biomass was observed upon exposure to W & H cleaning solution with a median of 1.1 (range 0.4 – 1.9) observed (Figure 7-8).

After 16h growth, the biomass of the *P. acnes* isolates was a median of 0.9 (range 0.4 – 1.6) (Figure 7-9). This was significantly reduced by Alconox®, Haemo-sol® and Rapizyme® ($p<0.001$) compared to exposure to RO H_2O alone

which reduced the biomass to a median of OD_{570} 0.5 (range 0.1 – 1.7) (Figure 7-9). Upon exposure tocleaning solutions, Alconox® reduced the biofilm biomass to a median OD_{570} of 0.2 (range 0.008 – 1.447), Haemo-sol® reduced the biomass to a median OD_{570} of 0.3 (range 0.02 -1.5) and Rapizyme® reduced the biomass to a median OD_{570} of 0.2 (range 0.02 – 0.8) (Figure 7-9). W & H cleaning solution did not result in a significant reduction of biofilm biomass with exposure resulting in a median OD_{570} of 1.0 (range 0.3 – 1.7) (Figure 7-9).

After 16h growth, the untreated *S. epidermidis* biomass from all experiments was calculated as a median OD_{570} of 1.3 (range 0.8 – 1.5) (Figure 7-10). This was reduced, though not significantly to a median OD_{570} of 1.179 (range 1.0 – 1.3) when exposed to the ROH_2O control (Figure 7-10). Exposure of the biofilm to Haemo-sol® detergent resulted in a significant ($p < 0.05$) reduction in the biofilm biomass to a median OD_{570} to 0.3 (range 0 – 0.9) (Figure 7-10). Exposure to Alconox® (median OD_{570} 0.3, range 0 – 1.264) and Rapizyme detergent (median OD_{570} 0.5, range 0.1 – 1.2) resulted in a reduction in biomass though this was not significant (Figure 7.11). W & H cleaning solution (median OD_{570} 0.89, range 0.4 – 1.3) did not result in a reduction of biofilm biomass (Figure 7-10).

The cell viability of untreated and treated *P. acnes* and *S. epidermidis* biofilms grown after 16h was assessed by measurement of the FU's caused by the reduction of alamarBlue® by live bacteria. For *P. acnes* biofilms, exposure to ROH_2O resulted in a non significant decrease in alamarBlue® reduction (median 101892 FU, range 39149 – 203732 FU) compared to the untreated control (median 231628 FU, range 149029 – 259453 FU) (Figure 7-11). Exposure to Alconox® (median 8818 FU, range 4308 – 44651), Haemo-sol® (median 10599 FU, range 4293 -42039 FU) and W&H cleaning solution (median 9288FU, range 4430 – 41558 FU) resulted in a significant ($p<0.001$) decrease in alamarBlue® reduction by biofilms (Figure 7-11). Exposure to Rapizyme® (median 17390 FU, range 4602 – 125952) also resulted in a significant ($p<0.05$) decrease in alamarBlue® reduction (Figure 7-11). All detergents decreased the alamarBlue® reduction to negative control levels (Figure 7-11).

For *S. epidermidis* biofilms, exposure to ROH_2O resulted in no change in alamarBlue® reduction (median120175 FU, range 106797 – 141772 FU) compared

to the untreated control (median 107990 FU, range 104838– 149006 FU) (Figure 7-12). Exposure to Alconox® (median 5028 FU, range 4892 – 9411), Haemo-sol® (median 4777 FU, range 4617 - 7133 FU), Rapizyme® (median 7284 FU, range 4889 – 35345) and W&H cleaning solution (median 4948 FU, range 4686– 5412 FU) resulted in a significant ($p<0.001$) decrease in alamarBlue® reduction by biofilms (Figure 7-12). All detergents decreased the alamarBlue® reduction to negative control levels.

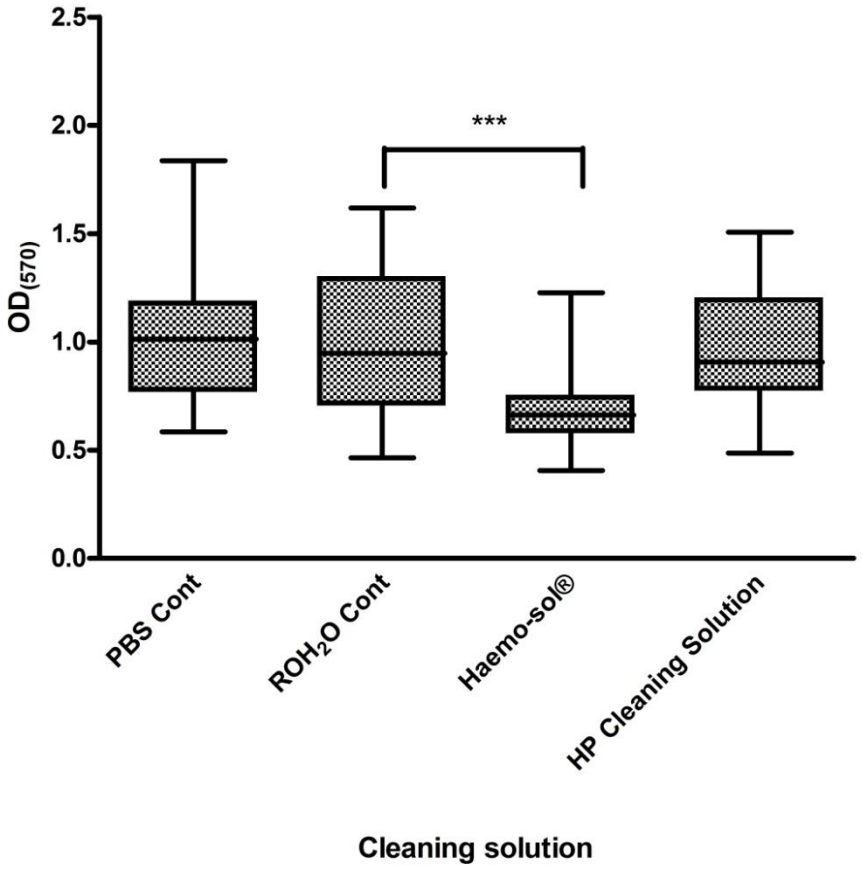

Figure 7-7 Total *P. acnes* biofilm biomass after treatment of cleaning solutions. *P. acnes* isolates from dental HP's were grown for 72h anaerobically in a 96 well plate to form biofilms. The biofilms were then exposed to cleaning solutions for 16 min and the biomass remaining after 3 rinses with PBS was measured using 1% crystal violet staining. Biofilms treated with Haemo-sol® have significantly (***= $p<0.001$) less biomass than RO H_2O alone. There was no significant difference between biofilms treated with the PBS control and the W & H cleaning solution. Data shown is the spread of all *P. acnes* isolates

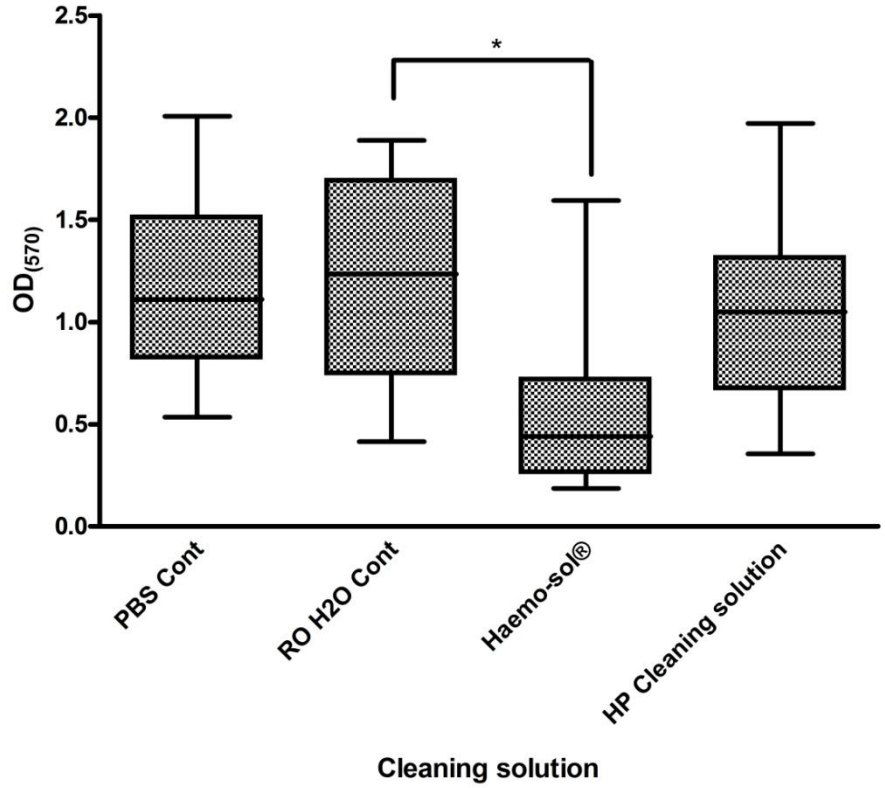

Figure 7-8 Total 72h *S. epidermidis* biofilm biomass detected after treatment with cleaning solutions. *S. epidermidis* strain RP62A was grown for 72h anaerobically in 96 well plates to form biofilms as a positive control for *P. acnes* biofilm formation. The biofilms were then exposed to cleaning solutions for 16 min and the biomass remaining after 3 rinses with PBS was measured using 1% crystal violet staining. Biofilms treated with Haemo-sol® detergent have significantly (*= $p<0.05$) less biomass than when exposed to RO H_2O alone. There was no significant difference between biofilm biomass treated with the PBS control and the W & H cleaning alcohol. Data shown is the spread of all *S. epidermidis* experiments.

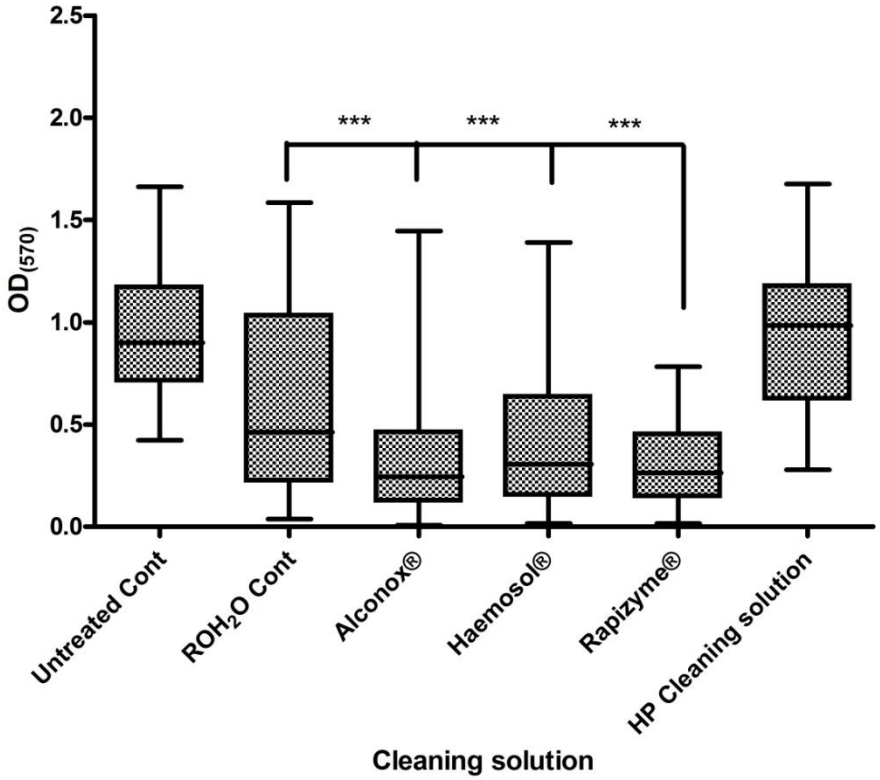

Figure 7-9 Total 16h *P.acnes* biofilm biomass detected after treatment with cleaning solutions *P. acnes* isolates from dental HPs were grown for 16h aerobically in a 96 well plate to form biofilms. The biofilms were then exposed to cleaning solutions for 16 min and the biomass remaining after 3 rinses with PBS was measured using 1% crystal violet staining. Biofilms treated with Alconox®, Haemo-sol® and Rapizyme® have significantly (***= $p<0.001$) less biomass than exposure to ROH_2O alone. There was no significant difference between biofilms treated with the ROH_2O control and the W & H cleaner. Data shown is the spread of all *P. acnes* isolates.

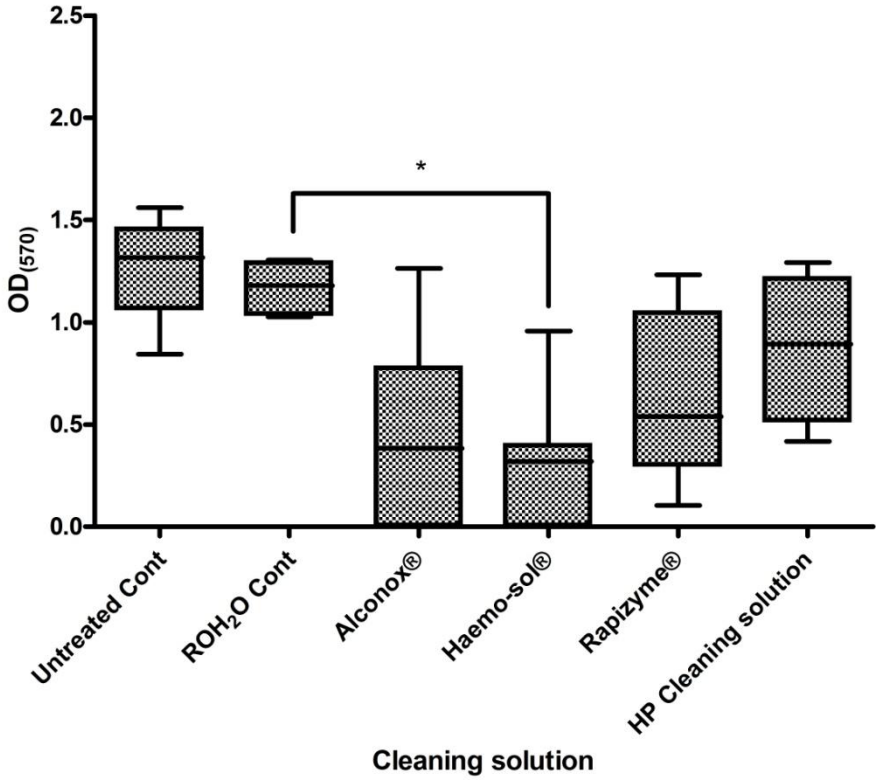

Figure 7-10 Total 16h *S. epidermidis* biofilm biomass detected after treatment with cleaning solutions. *S. epidermidis* strain RP62A was grown for 16h aerobically in 96 well plates to form biofilms as a positive control for *P. acnes* biofilm formation. The biofilms were then exposed to cleaning solutions for 16 min and the biomass remaining after 3 rinses with PBS was measured using 1% crystal violet staining. Biofilms treated with Haemo-sol® detergent have significantly (*= $p<0.05$) less biomass than when exposed to RO H_2O alone. There was no significant difference between biofilms treated with the RO H_2O control and biofilms treated with Alconox®, Rapizyme® and the W & H cleaner. Data shown is the spread of all *S. epidermidis* experiments.

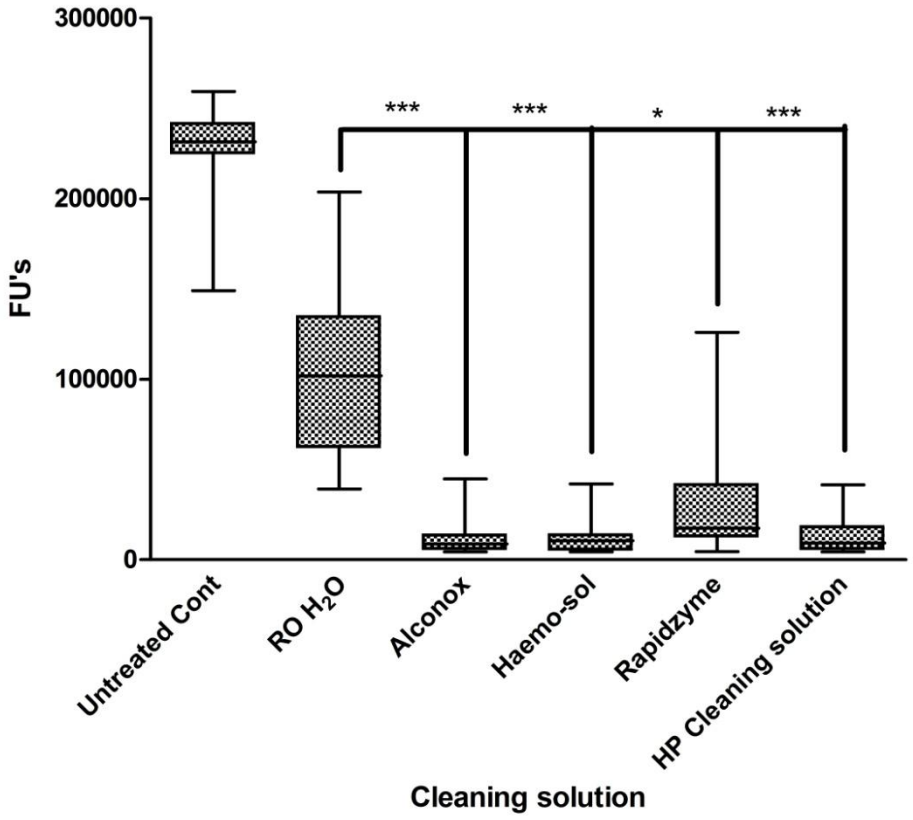

Figure 7-11 Bacterial cell viability of 16h biofilms formed by *P. acnes* isolates treated with cleaning solutions. Biofilms formed by *P. acnes* isolates from dental handpieces were tested for bacterial cell viability after exposure to the cleaning solutions and after 3 rinses with PBS by measuring alamarblue® reduction. Biofilms treated with Alconox®, Haemo-sol® and W & H cleaner have significantly (***= $p<0.001$) less viability than exposure to RO H_2O alone. Treatment with Rapizyme® also results in a significant (* = $p<0.05$) reduction in cell viability. All detergents reduced cell viability to negative control levels. Data shown is the spread of all *P. acnes* isolates.

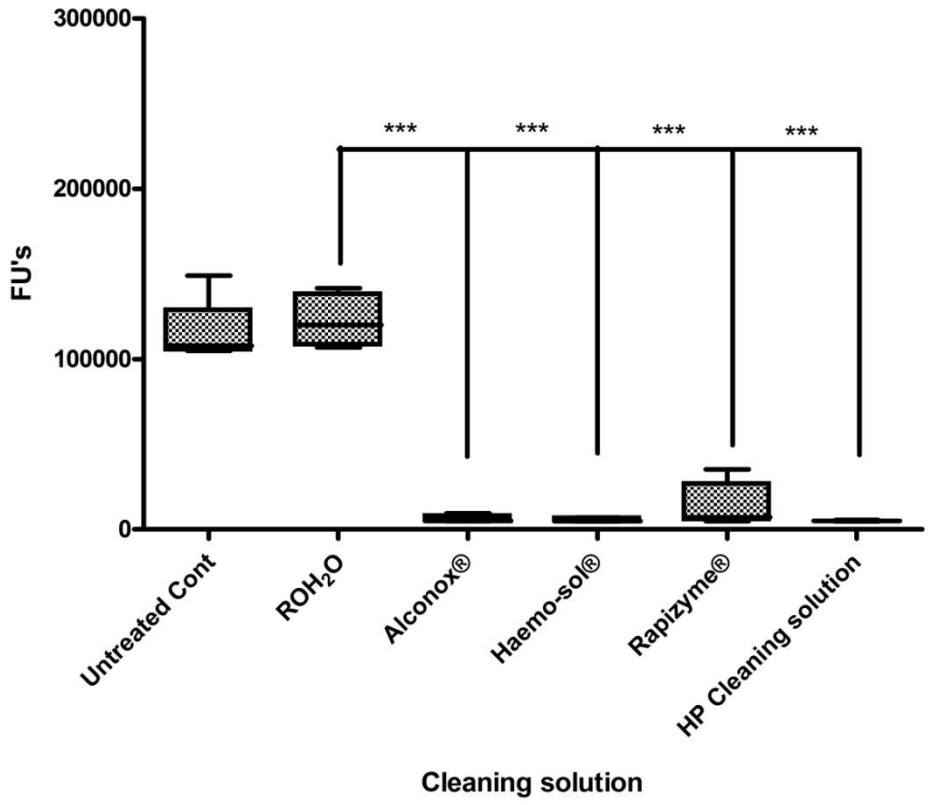

Figure 7-12 Bacterial cell viability of 16h biofilms formed by *S. epidermidis* RP62A treated with cleaning solutions. Biofilms formed by *S. epidermidis* were tested for bacterial cell viability after exposure to the cleaning solutionsand after 3 rinses with PBS by measuring alamarblue® reduction. Biofilms treated with Alconox®, Haemo-sol®, Rapizyme® and W & H cleaner have significantly (***= $p<0.001$) less viability than exposure to RO H_2O alone. All detergents reduced cell viability to negative control levels. Data shown is the spread of all *S. epidermidis* experiments.

7.2.7 *Efficacy of in vivo handpiece cleaning processes*

For the validation of the HP cleaners, A total of 7 WA- 99 contra angle HP's, 7 TA – 98 high speed HP's, and 7 WS-75 surgical HP's were sampled for each cleaning process. Of these, 3 of each HP were used as positive controls defined as a soiled HP that had not been subjected to cleaning and 1 of each HP was used as a negative control defined as an unsoiled handpiece put through each cleaning process. The HP test soil was based on the Swedish test soil detailed in BS ISO/TS consisting of citrated horse blood recalcified with O.1M $CaCl_2$ by adding a total of 100 µl of $CaCl_2$ was added to 900 µl of citrated blood(Zuhlsdorf et al., 2002). The blood protein concentration was based on the maximum protein concentration found on each handpiece part during the handpiece study (Chapter 5). Each dilution was performed in a solution of 400 µg/ml of salivary mucin representing the estimated physiological concentration in human saliva (Rayment et al., 2000). For the high speed TA- 98 HP's and the contra-angle WA – 99 HP'S, the blade of the turbine and the internal surface of the push button were inoculated with 20 µl of test soil equivalent to 20 µg of blood protein diluted in salivary mucin and each spray channel was contaminated by 10 µl of test soil in each tube equivalent to 40 µg of blood protein diluted in salivary mucin. The outer sleeve was contaminated with 50 µl of test soil. Additionally the middle gear of the WA-99 HP's was contaminated with 20 µl of test soil equivalent to 20 µg of blood protein diluted in salivary mucin. For the WS – 75 HP's, the turbine and the middle gear was contaminated with 20 µl of test soil equivalent to 60 µg of blood protein diluted in salivary mucin and the outer sleeve was contaminated with 50 µl of test soil equivalent to 150 µg of blood protein diluted in salivary mucin. HP's were then dried at ambient room temperature of 25°C for 15 min prior to cleaning with process 2 and for 60 min prior to cleaning by process 1. After cleaning, each HP part was aseptically dismantled and each component was added to a centrifuge tube and immersed in a measured volume of 1% (v/v) SDS. Each tube was added to a boiling water bath and boiled for 10 min at 100°C. For spray channel sampling, each lumen was rinsed once with 1 ml of boiling 1 % (v/v) SDS for a total sample volume of 2 ml. Each sample was measured using the OPA assay with comparison to a BSA standard curve.

No protein was detected from the negative control parts. For the reprocessed HP's, residual protein was isolated from a turbine (21.88 µg) and spray channels (54.5 µg) of 1 TA-98 HP (Table 7-8), the head gear of a WA-99 HP (76.4 µg) the outer sleeve of a WA-99 HP (2.29 µg) (Table 7-9), and outer sleeves of 3 WS-75 HP's and the middle gear of a WS-75 HP (median 370.4 range 321.4 – 387.6) (Table 7-10). No protein was detected from 2 TA -98 HP's and 2 WA-99 HP's (Table 7-8,Table 7-9). For the HP's reprocessed using process 1-1, protein was isolated from all WS-75 HP's (Table 7-10). Protein was isolated from the 3 turbines (median 36.9 µg range 29.37 – 42.31 µg), the 2 middle gears (median 59.8 µg, range <5 – 89.66 µg), and 2 outer sleeves (median 126.3 µg, range <5 – 339.8 µg) of the WS -75 HP's (Table 7-10). For the HP's reprocessed using process 1-2, protein was isolated from the turbine (91 µg) and the middle gear (2.3 µg) of 1 WS- 75 HP (Table 7-10). When HP's were reprocessed using process 1-3, protein was isolated from the headgear of 1 TA-98 HP (110.7 µg) (Table 7-8). No protein was isolated from the TA-98 HP's and WA-99 HP's reprocessed with process 1-1 and 1-2 and no protein was isolated from WS-75 HP's reprocessed with process 1-3 (Table 7-8, Table 7-9,Table 7-10).

Table 7-8 Protein isolated from TA-98 HP's before and after cleaning.

HP Part (n=3)	Median Protein before reprocessing (µg / part)	Process 1 -1 Median Protein concentration (µg / part)	Process 1-2 Median Protein concentration (µg / part)	Process 1-3 Median Protein concentration (µg / part)	Process 2 Median Protein concentration (µg / part)
TA-98 Turbine and Head gear	141.8 (Range 117.8 – 193.5)	<5	<5	<5 (Range <5 – 110.6)	<5 (Range <5 – 21.88)
TA-98 Spray channel	107.3 (Range 77.62 – 135.9)	<5	<5	<5	<5
TA-98 Outer Sleeve	216.1 (Range 130.9 – 326)	<5	<5	<5	<5 (Range <5 – 54.53)

Table 7-9 Protein isolated from WA-99 HP's before and after cleaning.

HP Part (n=3)	Median Protein before reprocessing (µg / part)	Process 1-1 Median Protein concentration (µg / part)	Process 1-2 Median Protein concentration (µg / part)	Process 1-3 Median Protein concentration (µg / part)	Process 2 Median Protein concentration (µg / part)
WA-99 Turbine and Head gear	192.7 Range (138.9 – 237.3)	<5	<5	<5	<5 (Range <5-76.15
WA-99 Middle Gear	147.4 Range (101.5-198.2)	<5	<5	<5	<5
WA-99 Spray channel	164.9 Range (114.8 – 298.2)	<5	<5	<5	<5
WA-99 Outer Sleeve	122.5 Range (101.2-183.5)	<5	<5	<5	<5 (Range 0 – 2.290)

Table 7-10 Protein contamination of WS-75 HP's before and after cleaning

HP Part (n=3)	Median Protein before reprocessing (µg / part)	Process 1-1 Median Protein concentration (µg / part)	Process 1-2 Median Protein concentration (µg / part)	Process 1-3 Median Protein concentration (µg / part)	Process 2 Median Protein concentration (µg / part)
WS- 75 Turbine and Head gear	370.8 Range (187.3-589.2)	36.91 (Range 29.37 -42.31)	<5 (Range <5 – 91.07)	<5	<5
WS-75 Middle Gear	355.6 Range(117.8 – 567.4)	59.8 (Range <5-89.66)	<5 (Range <5-2.297)	<5	<5 (Range <5-335.7)
WS-75 Outer Sleeve	488.4 Range(403.9 – 600)	126.3 (Range <5 - 339.8)	<5	<5	370.4 (Range 321.4 – 387.6)

7.3 Discussion

The cleaning of dental HP's, like other surgical instruments, is necessary to allow the removal of organic soil and protein contamination that may not be removed or may inhibit the sterilization process (Lipscomb et al., 2007). These include blood contamination and the bacterial biofilm. For dental HP's, serum albumin was detected in used, unprocessed HP samples which is the most abundant protein in blood (Desroches et al., 2007) (Chapter 5). This finding, along with visible blood on the HP's, indicates that blood is a typical contaminant of HP's before reprocessing. The current decontamination method used in the GDH first uses cleaning in an AWD followed by the Assistina cleaning machine (Table 2-3). The Assistina utilises an alcohol based cleaning solution and alcohol has been shown to fix any blood and protein to surfaces (Prior et al., 2004). To represent blood contamination, the Swedish test soil recommended by BS-ISO/TS for washer disinfector validation was used (Michels, 2008).

Bacteria growing on a surface are known to form a biofilm matrix to allow adsorption and increased survivability of bacterial cells (Coenye et al., 2007). The physical biofilm barrier is composed of proteins, DNA, and various structures that may inhibit sterilization of the bacterial cells and therefore disruption of the biofilm structure is carried out by the cleaning stage of the instrument decontamination process (Mah and O'Toole, 2001, Das et al., 2009). In dental HP's the majority of bacteria isolated were CONS and *P. acnes*, and these were isolated from various locations especially from the surgical HP (Chapter 4). Therefore, an *in vitro* system was utilised to measure the disruption of biofilms of *P. acnes* isolates and an *S. epidermidis* type strain known for the ability to form biofilms and to represent the CONS (Buckingham-Meyer et al., 2007, Polonio et al., 2001).

For the validation of *in vivo* cleaning processes, new HP cleaning machines were evaluated for cleaning efficacy. A total of 4 processes were sampled and compared to aid in product development. Process 1 has several cleaning stages lasting over 1 min with water being used as a cleaning solvent and a disinfection stage where detergent is introduced and process 2 involves a forced air containing detergent and lubricating oil lasting 4 seconds through the spray

channel and out of the head before the application of detergent onto the sleeve of the HP for external cleaning.

For *in vitro* cleaning validation, the effect of the four cleaning parameters described by the Sinners circle, cleaning time, temperature, force and cleaning solutions on the removal of blood protein were assessed. This utilised an *in vitro* tilting platform to provide an adjustable, reproducible cleaning force (Prior et al., 2004). There is currently no European standard to test for cleaning efficacy which meant this experiment was based on some of the instructions of BS-EN-ISO-15883 1-6 to test cleaning efficacy. The test soil was selected to represent a typical instrument contaminant as opposed to a harder to remove test soils (Desbuquois et al., 2010). The blood was left on the surface for 16h to represent the longest time it can take for an instrument to begin a decontamination cycle (Prior et al., 2004). Previous studies have shown the effects of drying time on cleanability of surfaces with a longer drying time representing a greater challenge for cleanability (Lipscomb et al., 2007). Blood protein removal was measured using the BCA assay, recommended by BS-EN–ISO-15883 part 1 to determine the protein concentration in solution. Knowing the blood protein concentration removed allows numerical and statistical comparison of blood removal. Previous studies have utilised the ninhydrin assay for the measurement of blood protein removal though this was to indicate the presence of protein rather than a quantitative measurement (Nakata et al., 2007). A thermostable adenylate kinase (tAK) has also been utilised for a quantitative analysis into the efficacy of washer disinfectors by measuring the enzyme removal on stainless steel (Hesp et al., 2010). Whilst this enzyme is represented as a model for prion protein with similar structure and behaviour, it does not represent the multiple proteins that are adsorbing to a surface *in vivo* (Hesp et al., 2010).

An increase in time and an increase in agitation speed result in an increase in blood protein removal and both represented an increase on the total cleaning force applied. An increase in the temperature of the cleaning solution results in an increase in the blood removal from the surface. The temperatures sampled represented ambient room temperature and elevated temperatures recommended by the detergent manufacturer's instructions (Table 7.1). In previous studies, a higher temperature of cleaning solution causes protein

denaturing and fixation to the surface (Crutwell, 2008, Desroches and Omanovic, 2008). These fixing effects in the case of the blood are observed at temperatures of 75 ^{O}C and this effect is found not to occur at temperatures closer to 60 ^{O}C (Crutwell, 2008).

The previous results from the studies of the 3 cleaning parameters were used to assess the efficacy of detergents recommended by the Scottish dental clinical effectiveness programme and additional detergents used in LDU's (Table 7.1). For further comparison, HiBi® Scrub, a chlorohexidine based antimicrobial hand wash and an example of a solution that is used in some surgeries for instrument cleaning was sampled along with 0.1M NaOH and W & H HP cleaning solution (Smith et al., 2009). NaOH has been recommended for reprocessing of instruments contaminated with the prion protein and W & H cleaning solution is alcohol based and currently used to process HP's (Kasermann and Kempf, 2003) (Table 2.2). The sampling time of 5 min and the agitation speed selected represent sub maximum levels of blood removal to allow comparison. The efficacy of each detergent at removing blood protein was first assessed at room temperature of 22^{o}C followed by the detergent manufacturers recommended temperatures if provided (Table 7-2). If a recommended temperature was not provided, a temperature was selected based on the instructions available for other detergents. For blood removal at room temperature, Haemo-sol® detergent removed the most blood though no cleaning solutions removed significantly more blood than the ROH_2O controls. Both HiBi® Scrub and the W & H cleaning solution removed significantly less blood highlighting the inadequacy of these products as instrument cleaners. Whilst there was variation of the mean percentage blood removal of the ROH_2O controls between experiments, the values were not statistically significant and changes in efficacy may have been due to variations in starting blood protein concentration.

To explain the action of detergents, the addition of an alkaline solution has been shown to reverse the interactions of serum albumin, the most abundant protein in blood, with a stainless steel surface by reversing the charge of the oxide layer that spontaneously forms on stainless steel (Sakiyama et al., 2004). Acidic solution has been shown to promote adsorption of serum albumin and may account for the low blood removal by the acidic HiBi® Scrub (Sakiyama et al.,

2004). Chlorohexidine, the main constituent of HiBi® Scrub has also been shown to precipitate the blood protein and not allow subsequent cleaning (Nakata et al., 2007). The addition of H_2O with a near neutral pH will also reduce adherence of BSA and change the conformation of surface bound proteins by internalising hydrophobic residues and increasing the surface area of bound proteins (Sakiyama et al., 2004).

It is not clear if the differences in detergent efficacy are due to the composition of the detergent due to the lack of information available from manufacturers. Treatment with alkaline detergents resulted in more blood protein removal than 0.1M NaOH showing that just having an alkaline solution alone cannot account for the cleanability shown. The detergents may also have different buffering capacities to ensure a stable pH compared to 0.1M NaOH alone. Previous studies have shown detergents are less effective at cleaning blood than water alone (Zuhlsdorf et al., 2002) and that detergents can fix blood to surfaces (Nakata et al., 2007). A study into the effect of detergents on blood showed the alteration of blood protein by fixation to the surface or the formation of precipitated protein (Nakata et al., 2007). Exposure to some detergents resulted in no protein removal upon subsequent treatment with an enzymatic detergent though blood was removed when no prior detergent immersion had taken place (Nakata et al., 2007). Cleaning using a washer disinfector removed all blood and this was attributed to the action of the water jets (Nakata et al., 2007). Knowledge of the ingredients of detergents may inform the development of detergents that can remove blood and protein contamination and improve the efficacy of the cleaning process.

The HP isolates that were studied were *P. acnes* isolated from S11 surgical HP's before decontamination and S11 surgical and TA-98 high speed HP's sent for repair (Chapter 4). Since not all *P. acnes* isolates had been type identified at the time of writing, no comparison was made between the type isolates and the ability to grow biofilms. The HP's for repair included documentation certificates indicating that they had been through a decontamination process though this could not be independently verified. A *S. epidermidis* strain was also sampled as a control and a representative of CONS isolated from dental HP's (Chapter 4). *P. acnes* is associated with the oral cavity and human skin where it can cause

benign inflammatory infections such as acne (Burkhart and Burkhart, 2007). *P. acnes* has been associated with bloodstream infections, the contamination of surgical implants and has been isolated from endodontic lesions (Ramage et al., 2003). If HP's used to treat these lesions were contaminated with *P. acnes,* the dental HP may act as a source of cross infection.

Currently, there are no reports on the detergent or other cleaning solution efficacy of removing *P. acnes* biofilms. Previous studies have focused on the efficacy of antimicrobials due to the growth of *P. acnes* on internal prosthetic hip joints and bone cement (Ramage et al., 2003). These studies have found that bacteria were more resistant to antibiotics due to the structure and the slow metabolic growth of bacteria which may not take up the antibiotic (Ramage et al., 2003). Similar mechanisms are evident for detergent resistance with *P. aeruginosa* biofilms resisting quarternary compounds by virtue of the ECM substrate and *S. aureus* hypothesised to resist through metabolic changes (Campanac et al., 2002). Further resistance mechanisms involve the hydrophilicity of the ECM surfaces, which may stop interaction of compounds with the ECM, and the inability of hydrophobic substances to pass through water channels (Campanac et al., 2002).

The aim of this study was to measure first the ability for *P. acnes* isolates to form biofilms under optimum and worst case scenario HP reprocessing conditions (Prior et al., 2004) before measuring the efficacy of detergents at disrupting the biofilm structure to allow the removal by subsequent rinses. This represents the disinfection and rinsing stages of the decontamination process. Biofilms were exposed to detergents for the same time as the disinfection stage of the decontamination cycle of the washer disinfector used in the Glasgow Dental School CSSD department (Table 2.2) and is the amount of time that contaminants would be exposed to detergents

When *P. acnes* and *S. epidermidis* isolates were grown under worst case instrument reprocessing conditions of 16h under aerobic conditions, all detergents sampled aside from the W & H cleaning solution resulted in a significant reduction in biofilm biomass when compared to RO H_2O alone. Again, the alkaline detergents Haemo-sol® and Alconox® were able to significantly

remove more biomass than RO H_2O alone as well as the enzymatic detergent which acts by digesting large organic molecules into water soluble dispersible fragments but is specific depending on the enzymatic target (Lequette et al., 2010). The W & H cleaning solution again failed to remove any of the biofilm material which further highlights the unsuitability of the W & H cleaning solution as a disinfectant.

Whilst each detergent was effective in disrupting the biofilm to allow subsequent removal, the W & H cleaning solution did not result in any change in biofilm biomass after rinsing, showing the fixing of the biofilm structure to the surface. This cleaning solution contains alcohol and this result is consistent with previous studies that have shown protein fixation when alcohol is applied (Prior et al., 2004). Haemo-sol® is an alkaline detergent and shows significant removal of the biofilm structure. The hydrated structure of the biofilm is known to allow small molecules such as alkaline detergents to move freely through the structure which will allow disruption which may denature the biofilm proteins or reverse the interactions of these proteins with the surface (Stewart et al., 2001).

The W & H cleaning solution reduced the bacterial cell viability to negative control levels which shows the penetration through the biofilm structure as the W &H cleaning solution does not remove the biofilm structure. Whilst the alkaline detergents also reduced the cell viability, the effect of the biofilm structure removal cannot be ruled out. The enzymatic detergent reduced cell viability but not to the same levels as the other detergents. This might be due to the neutral environment that the enzymatic detergent presents which will not result in *P. acnes* cell death.

Previous work on the efficacy of biofilm removal by cleaning solutions has studied the removal of *S. aureus* and *P. aeruginosa* with results showing increased removal by alkaline and acidic detergents and no difference in biofilm removal when a neutral detergent is applied. Whilst this is comparable to the results from this study, the actual cleaning was undertaken at high pressure and this may itself remove the biofilm without the application of detergent (Gibson et al., 1999).

Whilst the *in vitro* biofilm model has shown the efficacy of detergents at removing biofilms made by HP isolates, the model is not a complete biofilm model when in reference to dental HP's. The plastic of the plates does not represent the stainless steel handpiece surface which may have a different interaction with the biofilm proteins and their removal by detergents. The temperature to which the bacteria are exposed to in dental HP's is variable with heating coming during operation and cooling mediated by the dental unit waterline and may affect the growth of cells and the composition of biofilms. A surgical instrument biofilm may contain a number of different species which may explain the *P. acnes* isolates that were unable to form biofilms in the *in vitro* model.

For *in vivo* validation of HP cleaning, a novel test soil was applied based on previous findings (Chapter 6). The test soil comprised citrated horse blood with calcium chloride diluted in salivary mucin. Calcium chloride causes the citrated blood to coagulate and is similar to the behaviour of blood contamination *in vivo* (Crutwell, 2008). The addition of salivary mucin adds a protein associated with the oral cavity. Mucins are glycosylated proteins that are present on all mucosal surfaces and saliva (Rayment et al., 2000). The glycosylated structure gives them a viscous consistency that makes the test soil harder to remove (Rayment et al., 2000). The blood protein concentration was based on HP residual protein levels quantified (Chapter 5)

The test soil was applied to areas that protein was isolated from (Chapter 5). A number of internal parts and the external sleeve were inoculated to test the efficacy of each cleaning process at cleaning both internally and externally. The test soil was allowed to dry for 15 min for reprocessing using the future Assistina and 60 min before process 1 reprocessing to represent manufacturers instructions. The time of drying will have an impact on the adsorption of protein to the surface and therefore the cleanability of the instruments. Longer drying times have been found to make protein harder to remove during cleaning and therefore the shorter drying time used for future Assistina challenging may have resulted in a test soil that is easier to remove (Lipscomb et al., 2007).

A total of 3 different cleaning processes based on process 1 were sampled using the system. These were not defined and it is therefore not possible to speculate

on the differences between each process. Process 1-1 removed all protein inoculated on TA -98 and WA-99 HP's from all parts to limits below the sensitivity of the assay which would pass the BS-EN-ISO 15883 part 1-6 standards. For the WS -75 HP's, protein is found on all locations due to the increased protein concentration of the test soil. Similar findings were observed with process 1-2 with the protein reduced to limits below the sensitivity of the assay on the TA -98 and WA -99 HP parts. Protein was only isolated from 1 WS -75 HP which is an improvement on process 1-1. Process 1-3 removed protein to below the limits of the detection of the assay on all handpieces aside from 1 TA 98 HP and represents the most efficacious cleaning process of all tested.

Process 2 was found to remove all protein from all spray channels sampled but not the turbines of all TA -98 and WA-99 handpieces. This may be due to the location of the turbine as the last part to be contacted by the spray. There is also potential for contamination to be washed out the spray channels to settle on the turbine and cap if not rinsed thoroughly. The presence of residual protein on the outer sleeve also shows shortcomings in external cleaning. The surgical WS-75 HPs still had 75 % of the initial soil on the outer sleeves showing very little removal by process 2. The increase in test soil protein concentration and therefore the increasing challenge could not be removed by the future Assistina process.

The results suggest that process 1 is more effective at removing protein and mucin deposits than process 2. This can be explained using the Sinners circle which defines the key parameters of cleaning and their effects on the cleaning process. These parameters are time, cleaning chemicals, cleaning force, and temperature. Considering these factors, process 1 has a longer cleaning process, a greater cleaning force and a higher temperature than process 2 and therefore should present in theory a superior cleaning process. The cleaning chemicals are demineralised water for process 1 and detergent for process 2. Whilst no information is available on the composition of the detergent, the detergent may also fix blood protein to the surface.

The procedure employed to test the cleaning efficacy represents a more meaningful cleaning challenge due to the test soil based on typical HP contaminants. The total blood protein was lower than estimated due to an

inability to determine the initial concentration of blood protein. The salivary mucin diluent increased the protein concentration close to the mean levels previously isolated (Chapter 5). The components represent common HP contaminants but does not represent a complete picture of these contaminants. Bacteria are found before the decontamination process and any test soil may also contain a microbial element to make the test soil more realistic and harder to remove.

No single standard yet exists for "acceptable" protein levels on reprocessed instruments. The BS EN ISO–15883 part 1 defines an acceptable level as below the detection limit of one of three protein assays which are stated as 2 mg/m^2 for the Ninhydrin assay, 30 – 50 µg for the bicinchoninic acid assay, and 0.003 µmol of OPA sensitive amino groups for the OPA assay. Work undertaken by Lipscomb and colleagues (2006) also determined the threshold of sensitivity for similar reagents to be equivalent to 9.25 µg/ $10mm^2$for Ninhydrin and 6.7 µg/ $10mm^2$ for the Biuret test (Lipscomb et al., 2006a). If this was to be regarded as a threshold for cleanliness then both cleaning processes would fail this validation. A protein concentration of 200 µg/HP has also been suggested in the Zentral Steril 2008 Suppl Oct 16 as an upper limit threshold for cleanliness though this represents a greater protein concentration than was found in the HP research project and does not represent a realistic cleaning challenge.

It was not possible to undertake statistical analysis of the data due to the small sample size and number of replicates. Whilst this pilot study gives some idea of the efficacy of each cleaning process, firmer conclusions could be made by sampling more HP's.

These studies have described *in vitro* and *in vivo* systems to test the efficacy of cleaning solutions at removing common HP contaminants. These methods can be applied to the cleaning validation of other surgical instruments if the contaminants are known and can be isolated. For HP's, the study has also highlighted the unsuitability of an alcohol based HP cleaner which fixes the blood and biofilm and in the case of the biofilm, prevents subsequent removal. Whilst ROH_2O was as efficacious as detergents at removing blood protein, alkaline detergents such as Haemo-sol® resulted in biofilm structure removal and a reduction in bacterial cell viability. Due to the nature of *P. acnes* and the

abundance in HPs, any HP decontamination process should include *P. acnes* biofilm control measures. Further work is needed to characterise the multispecies biofilms containing other bacteria isolated from dental handpieces and methods of disrupting this biofilm to allow for HP decontamination.

For *in vivo* cleaning validation, all processes sampled were able to remove protein from internal and external HP parts to varying degrees. Process 1-3 resulted in the most HP parts with no detectable protein. An increased sample size for the same method will provide more robust conclusions and fully determine the efficacy of the machines at cleaning dental HP's.

8 Chapter 8: Discussion

8.1 Introduction

As early as 1924, Dental HP's were recognised as an infection control problem (Appleton Jr and L, 1924). This was before HP's derived rotary power by compressed air, had internal cooling systems based on the high pressure spray of water and before the HP had a more complex internal structure involving fibreoptic lights and other gears. After a public health scare in 1990 when patients were infected with HIV after dental treatment (Lewis et al., 1992), more focus was placed on decontamination procedures in dental clinics including the use of steam sterilizers and it was around this time dental HP's were referred to as "the weak link of infection control" when it was clear that contamination was able to enter the internal areas of the HP during use (Lewis et al., 1992). The source of HP contamination is from the oral cavity and the dental unit water line (Abel et al., 1971, Lewis et al., 1992). These contaminants can subsequently be released into patients when the compressed air and cooling water is operated again (Lewis and Boe, 1992). It is clear that despite advances in HP technology that aim to stop contamination entering such as anti retraction valves (Ozawa et al., Hu et al., 2007b), contamination is still able to enter the HP which has a more complex internal structure than in the past (Herd et al., 2007) therefore both the external and internal structures must be decontaminated after use.

The last published study into HP contamination *in vivo* was sampling low speed HP's for bacteria in 2007 (Herd et al., 2007). Therefore, contamination of HP's still occurs in more recent times.

8.2 Implications of findings

The aim of this study was to characterise the contamination of dental HP's before and after decontamination processes. To this end, the study characterised bacteria and protein contaminants before undertaking a detailed surface analysis of HP surfaces.

8.2.1 *Impact of research*

Though the dental HP has been shown to be contaminated with bacteria in the past, this study has found larger numbers of bacteria on each location and a larger number of species was also isolated than in previous studies by using a more sensitive extraction technique (Kellett and Holbrook, 1980, Dreyer and Hauman, 2001) (Chapter 4). This has increased the knowledge of the typical microbial contaminants and shown the advantages of sonication for the sampling of instrument contamination (Kellett and Holbrook, 1980, Dreyer and Hauman, 2001).

The most important finding in this study was the characterisation of protein contamination of dental HP's. This is so far the only study on HP's to apply quantitative analysis of HP parts by using the OPA assay that is recommended by BS-EN–ISO-15883 part 1 and used in previous instrument contamination studies (Smith et al., 2005, Vassey et al.). The method selected for desorption of protein was also based on BS-EN-ISO-15883 part 1 which recommends flushing of instruments with 1% (v/v) SDS. The method of extraction was changed to boiling of the sample to provide increased extraction efficiency and to allow reproducibility of the method. It was also essential to pick a method that will allow the protein to be visualised by SDS-PAGE with sacrifices made in protein extraction efficiency compared to previous studies (Baxter et al., 2006). Protein was found on all HP parts that had previously been shown to be contaminated by bacteria mainly the turbine (Kellett and Holbrook, 1980) and the spray channel (Dreyer and Hauman, 2001). Though the surgical HP inner gear has not previously been sampled in previous studies, it was assumed that this would be contaminated during use. The qualitative assessment of HP protein using SDS-PAGE was possible with the surgical gears upon precipitation of the samples. The main shortcoming with this method is that analysis is reliant on what contamination can be removed from the surface. To overcome this, a detailed surface analysis of HP surfaces using SEM, EDX analysis and a novel EFSCAN technique (Chapter 6). Contamination was detected on every surface sampled, including decontaminated surfaces. SEM and EDX analysis revealed additional contaminants including calcium based residue and contamination trapped in lubricating oil. Lubricating oil has been shown to inhibit sterilization of

contaminants by not allowing steam penetration (Lewis et al., 1992, Hegna et al., 1978). The EFSCAN analysis detected more median protein than had been isolated when contamination was desorbed from the surface (Chapter 5).

8.2.2 *Handpiece contamination – test soil development and implications for cleaning process*

Validation of washer disinfectors by the BS-EN-ISO-15883 part 1 requires the removal of a specific test soil by each cleaning process. A total of 7 test soils have been deemed suitable for testing cleaning of surgical instruments including rigid endoscopes all of which have different ingredients and numbers of ingredients. The soils can range from blood with clotting factors, such as in the case of the Swedish and Austrian test soil, to a multi-ingredient soil such as the German test soil containing semolina, butter, sugar, milk powder, and a suspension of *E. faecium* (Table 8-1). Though some studies have shown the reproducibility of the process in laboratory environments (Zuhlsdorf and Martiny, 2005), it is clear that the more complex a test soil, the harder it will be to remove and a machine unable to remove the German test soil may still be deemed suitable if it removes the less complex Austrian or Swedish test soil (Desbuquois et al., 2010).

Decontamination standards often require a process that can remove and sterilize the most difficult to remove soils and the most heat tolerant organisms. The times and temperatures of sterilization processes are based on the killing of a *G. stearothermophilus* spores that has greater heat resistance than microorganisms encountered in the medical environment. If a cleaning process can remove a formulation that represents more challenge than would be encountered during routine use and the process can sterilize *G. stearothermophilus* spores then the decontamination process will also clean and sterilize any challenge encountered routinely. There have previously been calls for test soils that are specifically designed for each instrument based on the highest concentration of contaminants found *in vivo* (Alfa et al., 1999). To illustrate this debate, a recent study of the cleanability of dental HP's contaminated with the Edinburgh test soil found that washer disinfectors were unable to clear the test soil from HP turbines (Walker et al., 2010). Whilst legally this would imply that the washer

disinfector used would be unsuitable for HP cleaning, the Edinburgh test soil may contains an estimated 3.1 g of protein that is significantly larger concentration than would be found in any HP sampled in this study. The use of a test soil that contained similar protein concentrations to that found *in vivo* (turbines 210 µg, spray channel 448 µg, surgical gear 1936 µg) may have resulted in the machine removing protein below the limit of detection and therefore would be suitable for routine cleaning of HP's.

If a test soil for HP's was to be developed, qualitative analysis showing the presence of serum albumin and salivary mucin give an estimate of the constituents. Serum albumin is the most abundant protein in blood and plasma (Desroches et al., 2007) and that combined with the mass spectrometry analysis of used, unprocessed surgical HP's indicates that an HP test soil will consist of blood. Salivary mucins are glycosylated proteins and a major constituent of human saliva (Hu et al., 2007a) indicating that a blood and saliva combination would make an appropriate test soil (Table 8-1)though the initial concentrations of protein added would be dependent on the applied location. Identification of the other proteins present in the HP eluent showed mainly blood constituents that further justifies the inclusion of blood in the test soil.

Some test soils including the German and the American also have a microbial constituent. This study indicates that the majority of bacteria from all locations were CONS (turbine $4x10^4$ CFU, spray channel $3x10^4$ CFU, surgical gear $1.7x10^5$ CFU) and a representative CONS would be suitable for incorporation into the test soil. Any test soil containing bacteria requires a 5 log reduction from a total starting concentration of $1x10^8$ CFU, which is how much bacteria would be present in a high speed and low speed HP test soil. The surgical gear has less total bacteria and a 5 log reduction would remove all the bacteria which may mean this minimum cleaning efficacy would require alteration. Surface analysis of dental HP's revealed contaminants immersed in lubricating oil (Chapter 7). The addition of lubricating oil to instruments has long been thought to inhibit sterilization of bacteria and removal of contaminants (Edwardsson et al., 1983). The addition of lubricating oil to the test soil may provide a greater challenge for contaminant removal though the object of the decontamination process is not to remove lubricating oil.

Knowledge of the HP contaminants allowed the development of *in vitro* models to test the cleaning efficacy of cleaning solutions (Chapter 7). For blood removal, no detergent based cleaning solution removed more blood than ROH_2O alone though alkaline and enzymatic detergents were able to reduce the biomass of biofilms made by HP isolates. A solution manufactured specifically for HP cleaning fixed the blood and the biofilm structure to the surface and its use should be discontinued and replaced with an alkaline detergent which combined significant biofilm biomass removal with blood removal comparable to ROH_2O alone. These tests can also be adapted to test the cleaning efficacy of other contaminants or test the efficacy of other cleaning solutions.

Table 8-1 Details of test soils for validation of surgical instrument cleaning and proposal for new dental HP test soil. Taken from BS-ISO/TS part 5

Country	**Constituents of test soil**
Austrian	Heparinized sheep blood coagulated with protamine
German test soils	• Sheep blood, *E. faecium* • Egg yolk, *E. faecium* • Semolina, butter, sugar, milk powder, *E. faecium*
Germany II	Tetramethylbenzidine hydrogen peroxide solution bovine haemoglobin
Netherlands	Bovine serum albumin fraction 5 porcine gastric mucin tye 3 bovine fibrinogen fraction 1 bovine thrombin
Sweden	Citrated cattle blood coagulated with calcium chloride
United Kingdom	Defibrinated horse blood/sheep blood, egg yolk, dehydrated hog mucin
Unites States of America	Protein/organic soil (user preference), *B. atrophaeus* endospores Albumin, haemoglobin, fibrinogen, thrombin
Proposed dental HP test soil	Coagulase negative *Staphylococci, P. acnes* biofilms, Citrated blood with $CaCl_2$ (Turbine concentration 200 µg, Spray channel concentration 400 µg, Surgical HP concentration (2000 µg). Salivary mucin.

8.2.3 *Handpiece contamination – cross contamination risks*

Very little bacteria and protein were isolated from HP's after decontamination using the GDH method (Section 5.2-10). No bacteria were isolated from turbines and surgical gears after decontamination using our extraction method (Chapter 5). Despite surgical gears having significantly more bacteria than turbines and spray channels before decontamination, surgical HP's are designed for simple disassembly to allow access of the inner gear for cleaning due to the invasive procedures carried out by the surgical HP. Bacteria were found in significantly smaller numbers in spray channels after decontamination. The bacteria identified were *P. acnes*, CONS and Gram negative bacilli which are associated with opportunistic infections of the oral cavity though these are not fatal (Jackson et al., 1999, Fujii et al., 2009, Aragone et al., 1992). The bacteria were not present in large numbers but the fact that bacteria are present indicates a failure of the GDH sterilization process and requires further investigation involving a larger sample size.

Refering to the BS-EN-ISO-15883 part 1 standard where a positive reaction with the OPA assay indicates a failure of the decontamination process, a total of 7 turbines, 5 spray channels and 1 surgical gear would fail this cleaning validation. The sensitivity of the OPA assay was calculated as 5 µg/ml of protein (Chapter 3) and opinion differs as to the cross contamination risk associated with protein cotamination (Lipscomb et al., 2006a). A protein based contaminant known to cause disease is the prion protein that causes Creutzfeldt- Jakob disease (CJD) which is a fatal neurodegenerative disease (Glatzel et al., 2003) whilst most protein contamination will only be benign. The prion protein is resistant to steam sterilization (Taylor and McConnell, 1988) and certain chemical inactivation methods (Mould et al., 1965). The risk of direct CJD transmission through dental procedures is rated as low according to recent department of health guidelines (Department of Health, 2007) with an estimated risk assessment and a study of oral and brain human tissue of CJD victims showed that only the brain and trigeminal ganglion contained any detectable prion protein (Head et al., 2003). No prion potein was detected in the alveolar nerve,

dental pulp, gingiva, salivary gland or tongue that are more likely to be contacted during dental surgery (Head et al., 2003). An animal model has shown the transmission of prion protein through intradental injection of the left incisor (Ingrosso et al., 1999). This represents a worst case scenario that shows that transmission through this oral route is possible. This current study did not attempt to identify the prion protein from HP samples but the presence of internalised blood protein indicating blood protein may be of concern due to the transmission of prion protein through blood transfusion (Flanagan and Barbara, 1996), (Llewelyn et al., 2004).

Surface analysis using SEM and EDX analysis reveals microscopic contamination on surfaces that had been through decontamination processes (Chapter 7). The impact of this contamination is unclear due to the limited identification of these contaminants. One issue highlighted was the presence of contaminants surrounded by lubricating oil which may have implications for the use of lubricating oil before decontamination as it may trap contaminants and prevent their removal. The use of lubricating oil should ideally follow the decontamination process to allow contaminants to be removed.

8.2.4 *Limitations of the study*

The survey of HP contaminants using desorption (Chapter 4, Chapter 5) was based on a few key HP parts such as the turbine that had been shown to be susceptible to contamination in previous studies (Lewis and Boe, 1992). It is conceivable that contamination may travel to other parts of the HP and be missed by the survey, such as the internal gear of low speed HP's(Chin et al., 2006). These additional inner parts and gears would have no direct contact with the patient even upon the passage of water or compressed air though an argument can be made that the entire HP must be sterile to assure a sterile instrument. The access of these parts would also require specialist dismantling of the HP that would cause it to cease to function or require extensive repair. Whilst swabbing of these parts could isolate bacteria and protein the current study preferred an approach with increased sensitivity as opposed to swabbing which has varying efficacies (Lipscomb et al., 2006a). Therefore a complete picture of HP contamination was not possible in this survey.

The HP's sampled had to return to the general circulation of the GDH and therefore could not be sacrificed for access to parts or to apply a more sensitive protein removal technique or to utilise certain surface analysis techniques involving chemical fixation of samples. Whilst this represented a cost saving for the study the fact that contaminants may be missed cannot be ignored. Ideally, a number of HP's would be sacrificed to allow a more complete analysis involving acid stripping or fixation of the instrument for visualisation of organic contamination on a surface using SEM. The high cost of HP's is a major obstacle to any studies of this nature and an alternative would be to sacrifice certain HP parts.

The bacteria isolated from HP's did not incorporate or identify any non culturable bacteria either due to their fastidious nature or the inability to grow on the media selected. A larger selection of media and application of 16S PCR sequencing will allow identification of additional isolates.

Protein isolated from 20 precipitated samples of turbines and spray channels before decontamination was at too low a concentration to be identified by the MS method (Chapter 6). Precipitation methods will inevitably result in a loss of some protein (Chapter 6) though were essential for this study due to the large sample volumes. The precipitation of more samples of turbines and spray channels may allow for a higher concentration of protein that is able to be identified by mass spectrometry. With the data available for the development of an HP test soil, it is assumed that turbines and spray channels are exposed to similar contaminants to the surgical gear albeit at lower protein concentrations.

8.3 Future work

Future work leading on from this research can be applied to study the contamination and decontamination of both HP's and other instruments. Dental practitioners, whom may have varied decontamination processes (Smith et al., 2009), provide the majority of dental services in the UK and the contaminants encountered may provide a more realistic contamination for a test soil. This would also inform on the optimum methods for the decontamination of dental HP's that can be applied to a dental practice setting that may not have the

resources and space to set up a decontamination facility that exits in a larger hospital.

A gap in this study was the sampling for viruses which are capable of being internalised and survive inside the HP (Epstein et al., 1995). Some blood borne viruses are capable of causing fatal disease if patient exposure occurs. For HP's *in vivo,* HIV and HBV DNA has been isolated after treatment of infected patients (Hu et al., 2007b, Lewis and Boe, 1992). The DNA does not indicate if the virus is viable and capable of causing disease. HP studies using PCR to highlight the presence of DNA coupled with cell culture infectivity studies would identify the potential viral contaminants as well as indicating the presence of viable viruses (Epstein et al., 1995).

There exists a lack of validated data justifying the use of AWD's for the cleaning of dental HP's which are recommended for routine decontamination. Whilst WD's are recommended as the gold standard for cleaning and is used in central decontamination facilities (Smith et al., 2009), recent studies have shown that they do not always result in an increase in protein removal when compared to ultrasonic baths or other methods of cleaning (Vassey et al., 2011). Therefore to justify guidelines for HP's, the efficacy of WD's should be measured much as the vacuum sterilizer has been validated through scientific study (Andersen et al., 1999). The surface analysis techniques detailed in this study can allow for the sensitive validation of decontamination processes (Baxter et al., 2009). By discovering and highlighting areas of the instrument that are contaminated during use, the subsequent surface analysis of the same locations after cleaning would show if the contaminants have been removed or if the cleaning process would require redesign to access certain areas.

The presence of CONS and *P. acnes* isolates in decontaminated HP's requires further investigation to determine the cross contamination risk. Both CONS and *P.acnes* have been isolated from endodontic pockets and are therefore associated with dental infections (Niazi et al., 2010). The link between dental HP's and infections can be proven by isolating bacteria from both the HP and any dental infection and sequencing any identical species recovered. This would

confirm an origin of dental infections as inadequately decontaminated dental HP's.

The HP is a mechanical tool that has a complex internal structure and requires lubrication to prolong the life of the HP and it is clear that both the structure and the lubricating oil are inhibiting decontamination processes. Whilst the cross contamination risk is still a long way from being fully explored, the design of future HP's should take decontamination into account. Recently, single use HP's have been developed to overcome the issues associated with decontamination of dental HP's.Whilst single use HP's would eliminate the cross contamination risk and the need for the development of decontamination processes; the cheap material used in the construction may not allow it to undertake some processes. The single use HP may also be associated with increased costs and increased waste that would be generated may make a single use HP currently unworkable. An alternative to single use HP's can be a fundamental change in HP design. This would require collaboration between researchers, HP manufacturers, engineers and perhaps manufacturers of other surgical PT's to inform development of an HP that can deliver an instrument of equal performance, provide cooling of the tooth surface, have as little moving parts as possible be easily dissasembled and include ceramic ball bearings to reduce or eliminate the need for lubrication. With this additional work, the dental HP may lose the unenviable title of the "weak link of infection control".

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